Enhancement of anti-tumour immunity by transduction with a *Mycobacterium tuberculosis* gene

Thesis

UNRESTRICTED

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ENHANCEMENT OF ANTI-TUMOUR IMMUNITY BY TRANSDUCTION WITH A *MYCOBACTERIUM TUBERCULOSIS* GENE

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ABSTRACT

As a strategy to enhance immune response against tumours by a "danger signal", the *Mycobacterium tuberculosis Ag38* gene encoding an highly immunogenic protein has been transduced in tumour cells. The gene was stably expressed on tumour cell surface by using a retroviral vector modified to express the leader and transmembrane sequences of the Nerve Growth Factor Receptor. Transduced cells have been used as a cellular vaccine in syngeneic mice and their ability to elicit an anti-tumour response has been evaluated against both a transplanted tumour model and against a spontaneous tumour model.

In the transplanted melanoma model, vaccination with transduced cells induced a significant protection against subcutaneous or intravenous challenge with non-transduced cells. In *Ag38*-transduced vaccinated mice a preferential Th1 response was observed. Moreover, after challenge, a high titre of antibodies directed against tumour cells was detected in protected mice. Most of these antibodies were directed against endogenously expressed viral antigens, while no reactivity against melanocyte lineage-specific antigens was observed.

In the HER2/Neu transgenic mice model, which spontaneously develop stochastic mammary tumours after a long latency period, the onset of tumour development was significantly delayed in mice vaccinated with *Ag38*-transduced cells. The delay in tumour development was increased when mice were vaccinated with the *Ag38*-transduced vaccine plus a systemic administration of IL-12 at a low dose. Consistent with melanoma model, a preferential Th1 profile was observed in mice in response to vaccination with Ag38-transduced cells and a $CD3^+CD8^+$ population able to respond to the tumour with IFN-γ production was derived from these mice. No humoral response

was induced in protected mice, while an activated $CD4^+$ T cell population producing IL-4 was obtained from long-survived mice.

These findings show the efficacy of a short-term protocol of vaccinations exploiting the adjuvant potency of a *Mycobacterium tuberculosis* gene. This adjuvant approach may represent a promising immunotherapeutic strategy for cancer immunisation.

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GLOSSARY OF ABBREVIATIONS

APC	antigen presenting cell
bp	base pair
BCG	Bacillus Calmette Guérin
cDNA	complementary deoxyribonucleic acid
CFA	complete Freund's adjuvant
CpG	cytosine-guanosine dinucleotide
cpm	counts per minute
CTL	cytotoxic T lymphocyte
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTPs	dATP, dCTP, dGTP, dTTP
DC	dendritic cell
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ds	double stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-1inked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
G	gramme
GM-CSF	granulocyte macrophage colony stimulating factor
HLA	human leukocyte antigen
HSP	heat shock protein
IFN-γ	interferon-γ
IL-	interleukin-
i. p.	Intraperitoneous
i. v.	intravenous
kb	kilobase
Kda	kilodalton
LAK	lymphokine-activated killer (cell)
LTR	long terminal repeat
MAb	monoclonal antibody
M	molar
MAA	melanoma associated antigens
MHC	major histocompatibility complex
MLV	murine leukaemia virus
MMTV	mouse mammary tumour virus
MoMLV	Moloney murine leukaemia virus
MOPS	-3-(N-morpholino) propanesulphonic acid
mRNA	messenger ribonucleic acid
NGFR	Nerve Growth Factor Receptor
NK	natural killer (cell)
PBLs	peripheral blood lymphocytes
pbs	primer binding site (on retroviral genome)
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PE	phycoerythrin
ppt	polypurine tract (on retroviral genome)
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute 1640 (cell culture medium)
RT-PCR	Reverse transcriptase-PCR
s.c.	subcutaneous
SSC	standard saline citrate
SDS	sodium dodecyl sulphate
ST-CF	short term culture filtrate (mycobacterial antigens)
TAA	tumour associated antigens
TAE	Tris-acetate-EDTA buffer
TCR	T cell receptor
TE	Tris-EDTA
Th	T helper
TIL	tumour infiltrating lymphocyte
TNF-	tumour necrosis factor-
Tris	tris[hydroxymethyl] aminomethane
v /v	volume per volume
w /v	weight per volume
X- gal	5-bromo-4-chloroindoy l-(3-galactosidase)

CHAPTER ONE

INTRODUCTION

1.1 TUMOUR ANTIGENS

Malignant transformation is the end result of altered expression of genes related to normal cell growth control and differentiation. Altered expression results from a variety of mechanisms including gene amplification, somatic DNA mutation and gene translocation. Each mechanism results in the expression of proteins that have the potential to serve as tumour antigens and thus as targets for immune therapy.

The molecular identification of tumour antigens, their immunodominant peptides, and the receptors that recognize them have placed studies of tumour immunology and immunotherapy in the mainstream of immunological research.

In the past decade different immunotherapeutical protocols have provided the first demonstration that immune reactions against cancer antigens can lead to the regression of invasive tumours in selected patients.

1.1.1 METHODS FOR THE DETECTION OF TUMOUR ANTIGENS

Three major techniques have been used to identify cancer antigens capable of eliciting cellular immune reactions in humans.

- The majority of human tumour antigens now known have been identified by a genetic approach based on the transfection of genomic DNA or cDNA libraries into cells expressing the appropriate MHC molecule. Positive clones expressing tumour antigens

are identified on the basis of the ability to stimulate cytokine release by human T cells with specific anti-tumour reactivity (Boon, 1993; Rosenberg, 1996) (Fig. 1.1).

- Biochemical approach have had more limited success in the identification of human cancer antigens. Attempts have been made to elute peptides from tumour cells or from MHC molecules purified from tumour cells and to detect fractions capable of stimulating anti-tumour T cells after pulsing purified fractions onto antigen-presenting cells. Triple quadrapole mass spectrometric techniques have then been used to sequence the minute quantities of peptides obtained (Cox et al., 1994). This approach has been severely limited by the need for custom-made, highly specialised equipment and the requirement that peptides be present in sufficient quantity to enable their identification by these physical techniques (Fig. 1.1).

- Each of the two techniques mentioned above is dependent on the prior availability of T cells capable of recognising tumour antigens, a requirement that often cannot be met. A third approach to the identification of tumour antigens has involved attempts to develop, by *in vitro* sensitisation techniques, T cells against candidate tumour antigens (Parkhurst et al., 1998). Genes encoding candidate tumour antigens have been transfected or transduced into antigen-presenting cells or synthesised peptides from candidate antigens based on known MHC-binding motifs have been pulsed onto antigen-presenting cells and used for *in vitro* sensitisation's of peripheral blood lymphocytes (PBLs) from cancer patients. T cells successfully generated *in vitro* against candidate antigens have then been tested for their ability to recognise intact tumour cells, the presence of such reactivity providing evidence that these candidate proteins represent tumour antigens (Fig. 1.2).

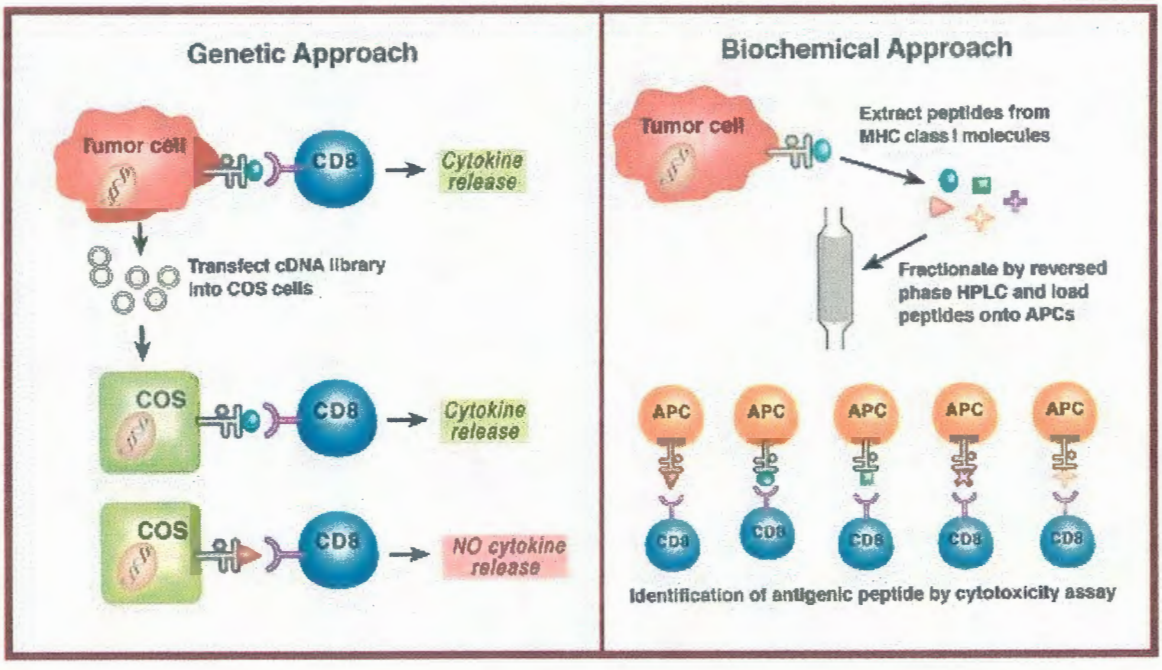


Fig. 1.1: Genetic and biochemical approaches to identify tumour antigens

Antigen-specific T cells are used to screen COS cells expressing a tumour-derived cDNA library (genetic approach) or peptides eluted from the tumour cells are pulsed onto APCs (biochemical approach) (from Greten et al., 1999).

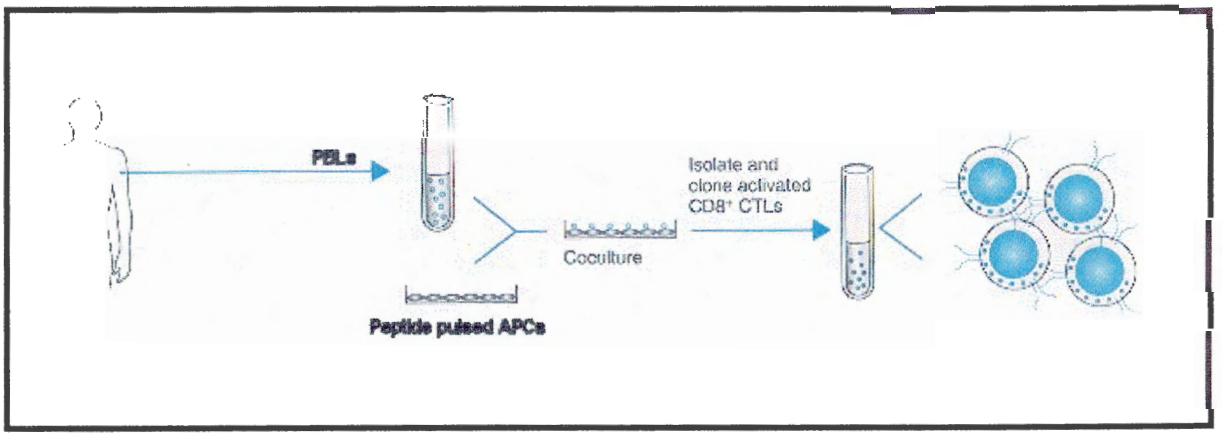


Fig. 1.2: Generation of tumour antigens-specific CTL clones

Peripheral blood lymphocytes (PBLs) from cancer patients are in vitro sensitised by co-culture with peptides from candidate antigens, based on known MHC-binding motifs, pulsed onto antigen presenting cells. Single T cells from these cultures are expanded into clonal CTL lines (modified from Abbas et al.).

For the identification of antibody-defined tumour antigens a technique has recently been described. This approach, called serological analysis of recombinant cDNA expression libraries (SEREX), uses the antibody repertoire of cancer patients for the systematic search for human tumour antigens. A cDNA library is constructed from fresh tumour specimens, packaged into lambda-phage vectors and expressed recombinantly in *Eschericha coli*. Recombinant proteins are transferred onto nitrocellulose membranes and identified as antigens by their reactivity with high-titre IgG antibodies present in the patient's serum. Reactive clones, detected using an enzyme-conjugated secondary antibody specific for human IgG, are recovered and subcloned to monoclonality. The nucleotide sequence of the inserted cDNA, which encodes the serological-defined tumour antigen, is determined by sequencing (Sahin et al., 1995; Chen et al., 1997) (Fig. 1.3).

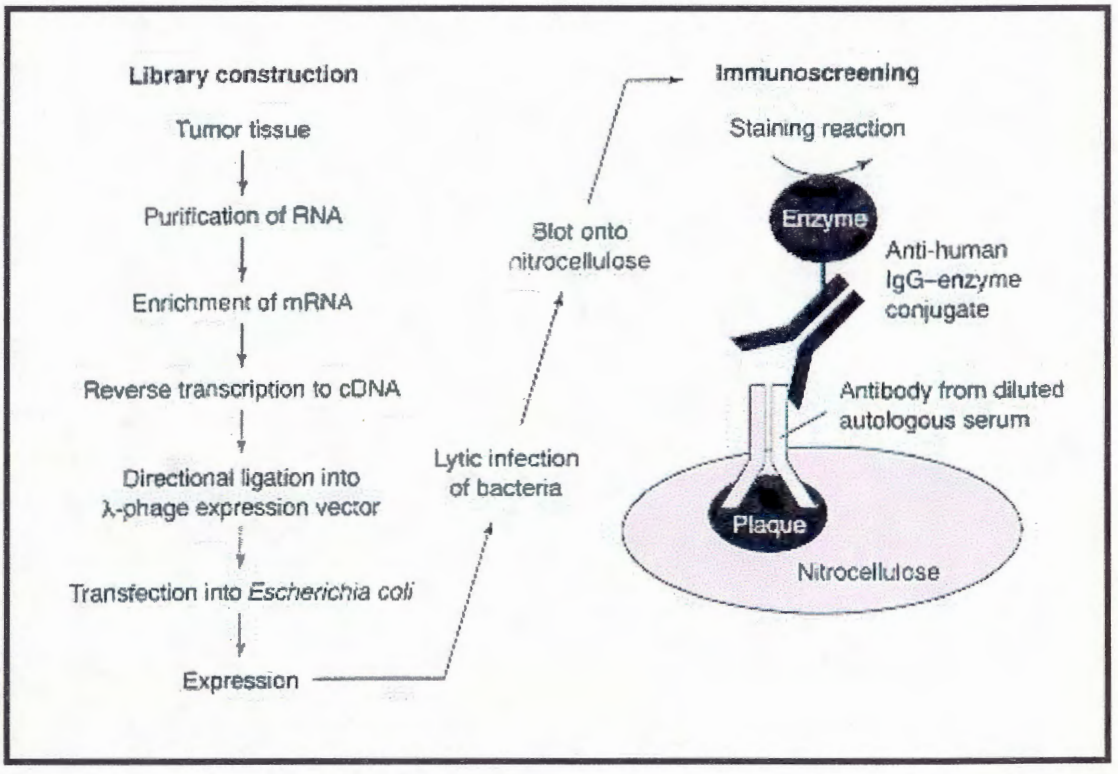


Fig. 1.3: Molecular definition of tumour antigens by the SEREX technique

A cDNA library is established from fresh tumour tissue and expressed in Escherichia coli using a λ-phage expression vector. The bacteria are grown in colonies and the lytic plaques are transferred by blotting to a nitrocellulose membrane. The diluted autologous serum is then incubated with the membrane and those antibodies that bind to tumour-derived antigens expressed in lytic plaques are made visible by enzyme-conjugated goat anti-human IgG antibodies (from Türeci et al., 1997).

1.1.2 CHARACTERISTICS AND CLASSIFICATION OF T CELL-DEFINED TUMOUR ANTIGENS

Based on the pattern of expression of the parent protein, tumour antigens can be classified into five major groups.

1.1.2.1 Tumour-specific shared antigens

This first group of tumour antigens represents those antigens encoded by genes that are completely silent in most normal tissues but are activated in a number of tumours of various histological types.

Prototype antigens of this group are those encoded by the gene P1A in the mouse (Van den Eynde et al., 1991a) and by the MAGE genes in humans (De Plaen et al., 1994). MAGE-1 and MAGE-3 genes were isolated as melanoma antigens recognised by CTL clones established from a single melanoma patient who was repeatedly immunised with mutagenized autologous tumour cells (Van der Bruggen et al., 1991; Gaugler et al., 1994; Van den Eynde et al., 1997). These genes belonged to a multigene family containing at least 12 different genes expressed not only in melanoma, but also in a variety of cancers, including breast, lung, prostate, bladder, laryngeal, head and neck cancers, and sarcomas. They are not detected in normal human tissues with the exception of testis (Takahashi et al., 1995).

By screening cDNA libraries utilising T cell clones from the same patient used to identify the MAGE gene family, another series of multigene families called BAGE (Boel et al., 1995) and GAGE (Van den Eynde et al., 1995b) have been identified. These gene products appear to have similar expression distributions to the MAGE gene family.

These proteins are nucleoproteins, whose biological function is not yet known. Their expression in cancer cells usually results from a demethylation of their promoters (de Smet et al., 1996). Epitopes from the MAGE, GAGE, and BAGE genes have been identified that are restricted by HLA-A1, -A2, -B44, and -CW16 (Traversari et al., 1992; Gaugler et al., 1994; Van der Bruggen et al., 1994; Van der Bruggen et al., 1994; Fleischhauer et al., 1996; Tanaka et al., 1997).

1.1.2.2 Tissue-specific differentiation antigens

Tissue-specific shared tumour antigens, such as tyrosinase, MART-1/Melan-A, gp100, TRP-1/gp75 and TRP-2, have been identified. These antigens are expressed in melanoma, normal melanocytes and retina, but not in other normal human tissues. They are localised in the melanosomes, which are specific organelles produced only by melanocytes and wherein melanin pigments are synthesised and deposited. It is not known why melanosomal proteins are highly immunogenic. The presence of precursors capable of reacting with normal non-mutated self-antigens within the tumour has suggested that the inflammatory microenvironment at the site of the tumour has resulted in the breaking of peripheral tolerance of these antigens. The role of CTLs against melanocyte differentiation antigens in melanoma rejection is not clear, but it is supported by the reported association of vitiligo, a local depigmentation of the skin resulting from melanocyte destruction, with prolonged patient survival and with spontaneous regression of melanoma (Rosenberg et al., 1996). The administration of TIL specifically recognising these tumour antigens to autologous patients along with IL-2 induced tumour regression. Thus, they may represent tumour rejection antigens.

Tyrosinase is the first member of differentiation antigens identified; it is a melanosomal protein with tyrosine hydroxylase, 3,4-dihydroxyindole (DOPA) oxidase and 5,6-dihydroxyindole (DHI) oxidase activity and represents a principal enzyme involved in

melanin synthesis. Tyrosinase was demonstrated to give rise to different peptides recognised by HLA-A1-, -A2-, -A24-, -B44-, and DR4- restricted T cells (Robbins et al., 1994; Anichini et al., 1993; Brichard et al., 1993; Wolfel et al., 1994; Kang et al., 1995; Brichard et al., 1996) and also to peptides that are presented by class II molecules to CD4+ T cells (Topalian et al., 1996). Interestingly, one of the naturally occurring epitopes recognised by HLA-A2-restricted CTL was found to have posttranslational deamidation of an asparagine residue (Skipper et al., 1996a). Adoptive transfer of one of the HLA-A24-restricted TIL into an autologous patient resulted in complete regression of tumour (Robbins et al., 1994), and, in addition, tumour regression was observed after immunisation with HLA-A2-binding tyrosinase epitopes (Jager et al., 1996).

MART-1/Melan-A presents in melanosomes of melanocytes and melanoma cells, but its biological function has not yet been identified (Kawakami et al., 1994a; Coulie et al., 1994; Kawakami et al., 1997). MART-1 was found to be an immunodominant melanoma antigen recognised by the majority of HLA-A2-restricted melanoma-reactive TIL (Kawakami et al., 1994b). The peptide MART-1_{27-35} was found to be the immunodominant common T-cell epitope recognised by CTL. The high immunogenicity of this epitope may be explained by its low HLA-binding affinity, resulting in less expression on the cell surface and less induction of tolerance to this epitope (Kawakami et al., 1996). It has also been suggested that crossreactive peptides on bacterial or viral proteins may be responsible for the high immunogenicity of MART-1 (Loftus et al., 1996). Although tumour regression was not observed in several patients who received the MART-1-reactive TIL, evidence of tumour regression was observed in some patients who were immunised with the MART-1 peptide in incomplete Freund's adjuvant in a clinical trial (Cormier et al., 1997).

Gp100 is a membrane glycoprotein in melanosomes and may be involved in the late steps of melanin synthesis. Gp100 was found to be recognised by the murine monoclonal antibody HMB45 that is commonly used for the diagnosis of melanoma in pathology laboratories (Kawakami et al., 1994a; Adema et al., 1993a). Gp100 appears to represent an immunodominant antigen recognised by many HLA-A1-, -A2-, -A3-, -A24-restricted CTL (Kawakami et al., 1994a; Bakker et al., 1994; Cox et al., 1994; Kawakami et al., 1995; Bakker et al., 1995; Tsai et al., 1997; Skipper et al., 1996b). Interestingly, in contrast to other gp100 epitopes which are nonmutated self peptides, an epitope presented by HLA-A24 was found to be derived from an incompletely spliced transcript present in melanoma cells as well as in normal cultured melanocytes (Robbins et al., 1997). A significant correlation between the clinical response to adoptive transfer of TIL therapy and the recognition of gp100 by TIL has been observed (Kawakami et al., 1995).

TRP1/GP75 is a melanosomal glyco-membrane protein with DHI-2-carboxylic acid oxidase activity involved in melanin synthesis. TRP-1 was previously identified as an antigen recognised by IgG antibodies in the serum of a melanoma patient (Mattes et al., 1983), suggesting the existence of CD4+ T-cells specific for this protein. TRP1 was demonstrated to be recognised by HLA-A31-restricted melanoma reactive TIL (Wang et al., 1995). Transfer of this TIL plus IL-2 to the autologous patient resulted in tumour regression (Topalian et al., 1987). In the murine B16 melanoma model, administration of antibody specific for TRP1 was shown to result in the rejection of B16 melanoma as well as the development of vitiligo (Hara et al., 1995).

TRP2 is a melanosomal enzyme with DOPAchrome tautomerase activity involved in melanin synthesis. TRP2 is a member of the tyrosinase-related gene family and shares 40% amino acid sequence identity with both tyrosinase and TRP1. A TRP-2 epitope of

the normal TRP2 protein was found to be recognised by both HLA-A31- and HLA-A33-restricted CTL (Wang et al., 1996). Interestingly, a mouse TRP2 was also identified as tumour antigen recognised by CTL reactive with the murine B16 melanoma (Bloom et al., 1997). Studies of the TRP2 protein represent an example of the use of the *in vitro* sensitisation techniques to identify new tumour antigenic epitopes. To identify HLA-A2-restricted TRP2 epitopes, multiple peptides were synthesised from the TRP2 protein based on HLA-A2-binding motifs. An HLA-A2-restricted T cell was *in vitro* generated against one of these TRP2 peptides, that was also capable of recognising HLA-A2+ melanomas (Parkhurst et al., 1998).

1.1.2.3 Tumour-specific mutated antigens

This third group of antigens correspond to peptides derived from regions of ubiquitous proteins that are mutated in tumour cells.

Three such antigens have been identified in melanomas. An HLA-B44-restricted T cell was identified that recognised the product of a gene termed MUM-1 (melanoma-ubiquitous mutated) (Coulie et al., 1995). The epitope recognised by this T cell spanned an intron/exon boundary and contained a single base mutation. Since both the normal and mutated peptides bound efficiently to the class I HLA-B44 molecule, but only the mutated form could be recognised by T cells, this indicated that the mutation appeared to have an effect on T cell recognition.

An HLA-A2-restricted CTL clone was found to recognise the mutated product of the cyclin-dependent kinase 4 (CDK4) gene (Wolfel et al., 1995). A single base mutation in this gene resulted in the substitution of a cysteine for an arginine in an anchor position of this peptide and altered the binding properties of this peptide to HLA-A2. One-hundred-fold lower concentrations of the mutant peptide were recognised, as compared

to the recognition of normal peptide. The CDK4 interacts with cyclin D and plays an important role in regulating cell cycle progression. The mutated CDK4 identified by the CTL had decreased kinase activity as well as decreased binding to the CDK4 inhibitor p16 (Wolfel et al., 1995), suggesting the involvement of this mutation in tumourigenesis.

A TIL that recognised an HLA-A24 melanoma antigen was found to recognise a mutated β-catenin gene product. A single point mutation that substituted a phenylalanine for a serine residue gave rise to a peptide epitope that was recognised at 1 milion times lower concentration than the normal peptide (Robbins et al., 1996). The same mutation in β-catenin was found in about 10% of all melanomas examined (Rubinfeld et al., 1997). The β-catenin mutation results in the stabilisation of the mutated protein, which favours the constitutive formation of complexes with transcription factors such as Lef-1. Constitutive β-catenin-Lef-1 complexes may result in persistent transactivation of as yet unidentified target genes, thereby stimulating cell proliferation or inhibiting apoptosis (Rubinfeld et al., 1997; Peifer, 1997).

Another mutation which may antagonise apoptosis was identified with CTLs specific for a human squamous-cell carcinoma (Mandruzzato et al., 1997). The antigen is encoded by a mutated form of the CASP-8 gene, which product is required for the induction of apoptosis through Fas and the tumour necrosis factor receptor 1 (Boldin et al., 1996; Muzio et al., 1996). A single point mutation in the normal stop codon resulted in the extension of the normal open reading frame of this gene by a region of 88 amino acids containing a T cell epitope. The ability of the altered protein to trigger apoptosis appears to be reduced relative to wild-type caspase-8.

It is surprising that none of the frequently mutated oncogenes or tumour-suppressor genes, such as Ras or p53, have been picked up as targets of CTLs raised against tumour cells, although antibody against the proteins was detected in cancer patients. Synthetic Ras or p53 mutant peptides have, however, been used to generate CTL or rejection responses against tumours carrying the corresponding mutant genes (Skipper et al., 1993; Peace et al., 1994; Noguchi et al., 1994; Abrams et al., 1996).

1.1.2.4 Overexpressed and ubiquitous tumour antigens

A number of CTLs raised against autologous tumour cells have been found to recognise antigens encoded by nonmutated genes expressed in both normal and tumoural tissues, although the CTLs appeared to lyse the tumour cells specifically (Robbins et al., 1995; Ikeda et al., 1997).

An HLA-A24-restricted TIL line reactive with HLA-A24 melanomas was used to isolate a previously undescribed gene called p15 (Robbins et al., 1995). Although Northern blots revealed that p15 was expressed in normal tissues, only tumour was recognised by this TIL. The mechanisms involved in this unique tumour recognition have not yet been elucidated.

The PRAME gene encoded an antigen overexpressed in many tumours and expressed in low levels in a variety of normal tissues. In this case, reactive HLA-A24- restricted T cells were identified only against tumours that have lost the appropriate HLA-Cw7 class I molecule. Indeed, further studies demonstrated that these T cells expressed natural killer (NK) inhibitory receptors that prevent lysis of HLA class I positive cells (Ikeda et al., 1997).

Another antigen that is expressed in some normal tissues and overexpressed in tumours is HER-2/neu, which is found at high levels in about 30% of breast and ovarian carcinomas. Her-2 is a transmembrane glycoprotein with tyrosine kinase activity and homology to the epidermal growth factor (EGF) receptor. In breast cancer, HER-2/neu overexpression was reported to be associated with aggressive disease (Slamon et al., 1987). IgG antibodies specific for Her2/neu and T-cell proliferative responses to Her2/neu proteins were detected in breast cancer patients, however the details of CD4+ T-cell recognition, including HLA restriction and involved epitopes, have not yet been determined (Disis et al., 1994). A peptide derived from HER-2/neu was found to be the target of lymphocytes infiltrating some HLA-A2 ovarian carcinomas (Fisk et al., 1995; Lustgarten et al., 1997). Recognition and lysis of ovarian cancer cells by CTLs were functional events also shown to correlate with the expression level of HER-2/neu in the tumour cells (Yoshino et al., 1994). Interestingly, breast and ovarian cancer-specific CTLs recognised the same epitope peptide (GP2) derived from the HER-2/neu protein in the context of HLA-A2 (Peoples et al., 1995).

Some CTLs directed against breast, ovarian and pancreatic carcinomas recognise an epitope of mucin (Jerome et al., 1993; Barratt-Boyes, 1996), a surface protein composed of multiple tandem repeats of 20 amino acids. Whereas in normal cells mucin is heavily glycosylated, in these tumours the peptide repeats are unmasked by underglycosylation, resulting in CTL recognition. Remarkably, this recognition, which depends on the presence of the multiple repeats, occurs in the absence of HLA restriction.

1.1.2.5 Viral antigens

Antigens derived from oncogenic viruses constitute another category of potentially useful tumour antigens. A number of viral antigens have been studied in detail on

virally-induced mouse tumours (Klarnet et al., 1989; Tanaka et al., 1988; Kast et al., 1989; Plata et al., 1987; Kast et al., 1991) and shown to be relevant for tumour rejection.

In humans, more than 90% of cervical squamous cancers are Human Papilloma Virus (HPV)-positive (50% were HPV16-positive). HPV proteins E6 and E7 that are persistently expressed in these cancer cells and play an important role in oncogenesis, are attractive tumour-specific targets. Tumour-specific CTLs have been elicited by *in vitro* sensitisation with E7 peptides presented by HLA-A2 (Ressing et al., 1995).

Epstein-Barr Virus (EBV) appears to be involved in oncogenesis of some cancers, a subset of Hodgkin's disease and immunoblastic B-cell lymphoma that develops in immunocompromised patients. CTL specific for EBV proteins including EBNA-2, -3A, -3B, -3C, -4, -6, and LMP2 have been demonstrated (Gavioli et al., 1992; Murray et al., 1992).

1.1.3 NATURE OF SEROLOGICALLY-DEFINED TUMOUR ANTIGENS

During the past few years, SEREX has been applied to a range of tumour types, including melanoma, renal cancer, astrocytoma, Hodgkin's disease, esophageal cancer, lung cancer, colon cancer, gastric cancer, breast cancer and prostate cancer. This survey has identified a large number of tumour antigens, more than 400, only a small fraction of which has been analysed beyond the initial sequencing stage. Approximately one third of the SEREX-defined genes are novel. Different categories of tumour antigens have been identified (Old et al., 1998).

1.1.3.1 Mutational antigens

Antigens encoded by mutated genes have been demonstrated only rarely by the serological approach. An example is a mutated p53 isolated from a case of colon cancer (Scanlan et al., 1998).

1.1.3.2 3p antigens

Three SEREX-defined antigens are encoded by the p21 region on chromosome 3, a region known to be a hot spot of genetic abnormalities in many cancer types. An example is the NY-LU-12 gene, defined as human lung cancer antigen recognised by autologous antibodies (Güre et al., 1998). Although mutation may likely be the underlying mechanism for the immunogenicity of these antigens, no mutations have been detected yet in the coding sequences of the 3p antigens.

1.1.3.3 Differentiation antigens

The classic differentiation antigen tyrosinase has been detected in SEREX (Sahin et al., 1997), demonstrating that some of the serologically identified antigens are also targets

for CTLs. Another example in this category is represented by the gastrointestinal tract-related differentiation antigen galectin 4, defined as human colon cancer antigen recognised by autologous antibodies (Scanlan et al., 1998).

1.1.3.4 Amplified and overexpressed antigens

Several antigens coded by amplified and overexpressed genes have been identified, indicating that antigen overexpression can led to immunogenicity by overriding thresholds critical for the maintenance of tolerance. Examples in this category are a new isoform of carbonic anhydrase in renal cancer (Sahin et al., 1995), aldolase A (Güre et al., 1998) and the translation initiation factor elF-4γ (Brass et al., 1997) in lung cancer and the new human galectin, galectin 9, in Hodgkin's disease (Tureci et al., 1997).

1.1.3.5 Viral antigens

A virus-encoded antigen that elicits an autologous antibody response is the env protein of the human endogenous retrovirus HERV-K10, which was found in a renal cell cancer (Ono et al., 1986; Tureci et al., 1997).

1.1.3.6 Splice-variant antigens

Splice variants are also likely to be an immunogenic trigger as has been shown for the Hodgkin's disease associated antigen, restin (Sahin et al., 1995) and the colon cancer associated antigen, NY-CO-38 (Scanlan et al., 1998).

1.1.3.7 Cancer-testis antigens

Cancer-testis antigens (CTA) represent an expanding family of tumour antigens that are expressed selectively in a varying proportion, 10-40%, of different cancers. In normal tissues expression is highly restricted, with testis being the sole or predominant site of

CTA expression. Three antigens in this category, MAGE , BAGE and GAGE initially were identified as targets for cytotoxic T cells. HOM-MEL-40/SSX2 (Sahin et al., 1995; Gure et al., 1997), NY-ESO-1 (Chen et al., 1997; Chen et al., 1997), SCP1 (Tureci et al., 1998) and CT7 (Chen et al., 1998) were uncovered by SEREX analysis. Seven genes or gene families now belong to the CTA category and, interestingly, four of them have been shown to be coded for by the X chromosome (MAGE, GAGE, SSX and NY-ESP-1). Since no evidence has been obtained for mutation or other gene rearrangements involving CTA-coding genes, with a single exception that involves SSX genes in a single case of synovial sarcoma (Crew et al., 1995), the likely explanation for expression of CTA in cancer cells is gene activation or derepression. MAGE expression has been correlated with the state of global hypomethylation generally associated with cancer and spermatogenesis (de Smet et al., 1996). However, this cannot be the whole explanation because CTA are not coordinately expressed and certain tumour types, such as colon cancer, rarely express known CTA. SCP1, the only CTA with a known function, is a synaptonemal complex protein involved in chromosome reduction during meiosis; it is intriguing to speculate what role aberrant expression of a meiotic protein in a somatic cell plays in the origins and progression of cancer. CT7 is the most recent CTA defined by SEREX. The CT7 gene encodes a protein with the COOH terminus highly homologous to the MAGE-10 and other MAGE genes over a 200 amino acid stretch. To find new members of the CTA family, SEREX analysis is being extended to screening expression libraries derived from normal testis and tumour cell lines expressing one or more of the known CTA.

In conclusion, a great number of tumour antigens have been identified on the basis of their recognition by immune system through both T cell and/or humoral responses. The

identification of such large number of antigens demonstrates that immune system potentially has the ability to recognise and destroy tumours.

1.2 IMMUNE RESPONSE AGAINST TUMOUR CELLS

The "immune surveillance" theory of Thomas (1959) was refined by Burnett in 1970, who formally postulated that the immune system constantly monitors the body for abnormal cells and destroys the majority of tumours before they become clinically manifest (Burnet, 1970).

Several lines of evidence of tumour-specific immunity have been provided. In animal models it has convincingly been demonstrated that the immune system can recognise and eliminate tumour cells, especially by induction of tumour-specific T cells in cases where viruses play a role in the transformation event (Bernards et al., 1983; Kast et al., 1989). Also from several types of human malignancies, T cells directed against autologous tumour cells have been isolated (Boon et al., 1994). In patients, spontaneous regression of tumours has been observed, most frequently in renal cell carcinoma and malignant melanoma (Oliver et al., 1992). Furthermore, in immunocompromised patients, such as patients with organ transplants or HIV-infected patients, an increased frequency of tumours is observed (Bouwes et al., 1991; Oliver et al., 1992).

1.2.1 SELF AND NON-SELF THEORY

Host immune surveillance was suggested a long time ago to play a crucial role in the control of tumour growth on the assumption that tumour cells are seen as foreign by the host (Thomas, 1959; Burnet, 1970). As tumours arise after embryogenesis, negative thymic selection of T cells, based on self-discrimination, does not occur. Newly arising tumours are eliminated as they originate and only the few lacking "neo-antigens" can escape immune surveillance and become clinically detectable. This theory, therefore,

postulated that most newly arising cancer cells are destroyed by a vigorous immune system. Only occasionally tumour cells, which do not express neo-antigens, escape immune surveillance. Therefore, these cells can divide undisturbed by the immune system and develop into observable tumour masses.

The original concept of immune surveillance is no longer accepted since the molecular characterisation of tumour associated antigens (TAA) has shown that, for the most part, they are nonmutated self-molecules. Furthermore, at this point the role of immune suppression in the development of various cancers has not been conclusively established. Therefore, complex interactions between host and tumour cells that could lead to tumour rejection through breaks of self-tolerance similar to those which develop in auto-immune pathology need to be considered (Marincola, 1997). Thus, because most TAA are represented by nonmutated self-molecules and TAA-specific CTL responses have been identified in patients, the growth of tumours in spite of this immune response represents a phenomenon of peripheral tolerance. This view assumes that a host TAA-specific CTL response plays a predominant role in the control of tumour growth. Such a predominant role is still controversial and other immune mechanisms are likely to participate in the control of tumour growth (Sogn, 1998).

1.2.2 DANGER MODEL

Fuchs and Matzinger (Fuchs et al., 1996) proposed an alternative model to explain the coexistence in some case of effectors and target cells in tissues without the development of tumour rejection. In contrast with the theory that immunological recognition of cancer cells reflects the capacity of the immune system to discriminate between self and nonself molecules, this model envisions that the immune system starts an immune

response by detecting tissue distress (danger). It is possible that cancer cells cannot effectively provide danger signals and consequently the default response of T cells in the tumour microenvironment is turned off.

This model best fits with the characteristics of melanoma associated antigens (MAA), most of which are nonmutated self-molecules (differentiation antigens) and with the indolent immune response to them in patients with melanoma. Furthermore, self-MAA specific CTL are easily generated *in vitro* from PBMC and TIL of patients with melanoma but not always do they significantly affect tumour cell growth *in vivo*. This discrepancy may reflect the lack *in vivo* of a second signal, which *in vitro* is provided in addition to a primary specific stimulus to induce MAA-specific CTL.

In this view, the majority of tumours do not "escape" immune recognition but simply survive in a favourable environment without triggering a response. In circumstances where some cells undergo damage, irrespective of an immunisation protocol, the host immune response switches from self-tolerance to self-recognition; "tumour autoimmunity" occurs and malignant lesions regress. Availability of HLA class I antigen restricted TAA epitopes, while adequate to allow recognition and lysis of tumour cells by activated CTL, may not be sufficient to induce CTL activation and proliferation in the absence of a second stimulus provided either by costimulatory molecules or by stimulatory cytokines (Matzinger, 1994). Because the requirement for secretion by CTL of IL-2 or other cytokines promoting their activation and proliferation are relatively high (Tsuji et al., 1992; Valitutti et al., 1996), CD4+ T cells may have to provide additional stimulation which could lead to amplification of CTL response and to tumour rejection.

1.2.3 EFFECTOR MECHANISMS IN ANTI-TUMOUR IMMUNITY

Virtually all of the effector components of the immune system have the potential to contribute to the eradication of tumour cells. It is likely that each of these effector mechanism plays a role in the control of tumour growth, but a particular mechanism may be more or less important, depending on the tumour and setting.

1.2.3.1 Antibodies

Two major mechanisms potentially might be involved in antibody-dependent tumour lysis. Complement-fixing antibodies bind to the tumour cell membrane and promote attachment of complement components that create pores in the membrane, resulting in cell disruption. An alternative mechanism is antibody-dependent cell-mediated cytotoxicity (ADCC) in which antibodies, usually of IgG class, form an intercellular bridge by binding via the variable region to a specific determinant on the target cell and via the Fc region to effector cells expressing Fc receptors.

The observation that sera from some tumour patients specifically recognise structures selectively expressed on autologous tumour, but not normal, cells provided strong evidence for the existence of a specific antibody response against tumour in cancer patients. Recently, by the SEREX approach a large number of tumour antigens have been identified using the antibody repertoire of cancer patients (Old et al., 1998).

The clinical significance of antitumour antibodies in cancer patients is largely unknown. The presence of p53 antibodies has been reported to be associated with a poor prognosis (Houbiers et al., 1995; Coomber et al., 1996), while the clinical significance of anti-HER-2/neu antibodies and antibodies to mutated Ras oncogenes remains to be determined (Disis et al., 1996). More patients must be analysed to determine whether

the development of antibodies to tumour antigens is associated with clinically relevant features.

1.2.3.2 T cells

The T cell response is unquestionably the most important host response for the control of growth of antigenic tumour cells; it is responsible for both the direct killing of tumour cells and the activation of other components of the immune system.

T-cell immunity to tumours reflects the function of the two T-cell subsets: class II-restricted T cells, which largely represent CD4 helper T (Th) cells that mediate their effect by the secretion of lymphokines to activate other effectors cells and induce inflammatory responses, and class I-restricted T cells, which largely represent CD8 cytotoxic T (Tc) cells that can also secrete lymphokines but mediate their effect mostly by direct lysis of tumour cells (Fig. 1.4).

The precise contribution of each T cell subset and T cell function to the antitumour response appears quite variable, but tumour-specific T cells from each subset are capable of mediating tumour eradication and have been detected in the peripheral blood of individual patients and in the cells infiltrating human tumours.

1.2.3.2 (1) Antigen presentation pathways and recognition by T cells

The classical MHC class I pathway allows endogenous proteins within the cytosol to be presented on the cell surface to immune cells. Proteins are processed within the cytoplasm by degradative enzymes, probably within proteolytic complexes termed proteasomes. These peptides are then ferried into the lumen of the endoplasmic reticulum (ER) by the transporter proteins associated with antigen presentation (TAP). The TAP also ensures that the peptides are of a suitable size, of about 8 to 10 amino acid residues, for binding with class I molecules within the ER.

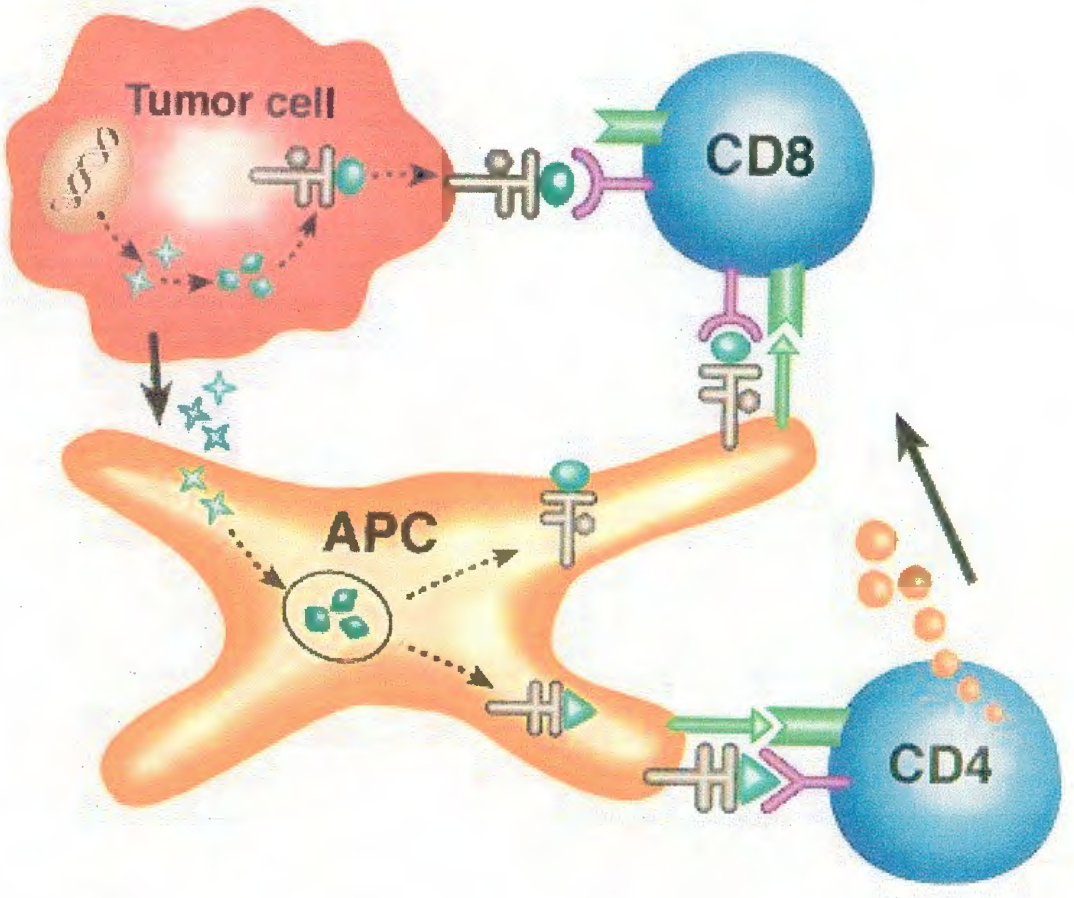


Fig. 1.4: T-cell immunity to tumours

Tumour cells present their antigens on the surface to $CD8^+$ T cells or release the antigen so that it can be taken up by professional APCs. These cells process the antigen and present it to $CD4^+$ and $CD8^+$ T cells by a mechanism called cross-priming. $CD4^+$ T cells provide cytokine help for $CD8^+$ T cells (from Greten et al., 1997).

The assembled peptide-MHC complex then proceeded to the cell's surface (Fig. 1.5). This pathway was first characterised for viral proteins synthesised by infected host cells (Germain, 1994). Most of the peptides presented in this manner are self peptides to which the immune system is tolerant; tumour antigens may be presented in a similar way on the tumour cell surface.

Recognition of the tumour antigen-MHC class I complex by a naive $CD8^+$ T cell bearing an appropriate TCR constitutes a signal for activation, but in order for the T cell to proliferate and mature into effective cytolytic T cells, the cell is dependent on various cytokines released by activated $CD4^+$ T helper cells.

A minority of tumours that express class II MHC molecules may directly activate tumour-specific $CD4^+$ helper T cells. More commonly, class II-expressing professional antigen-presenting cells (APCs) process and present internalised proteins derived from dying or phagocytosed tumour cells. APC cells include dendritic cells, which represent a widely-distributed heterogenous population of bone-marrow derived cells that have exquisite abilities of antigen presentation (Janeway, Jr. et al., 1994; Caux et al., 1995; Sprent, 1995), macrophages and activated B cells.

The MHC class II pathway of antigen presentation differs from that of MHC class I (Germain, 1994). In the ER, MHC class II molecules are precluded from binding peptides by the invariant chain. The MHC class II molecule proceeds to proteolytic intracellular compartments, such as lysosomes and endosomes, where the invariant chain is removed to allow binding of peptides derived from exogenous proteins which have been internalised from the extracellular space (Fig. 1.6).

1.2.3.2 (2) **MHC,** ***adhesion and accessory molecules***

The major histocompatibility complex, MHC, as described above, is required for antigen presentation.

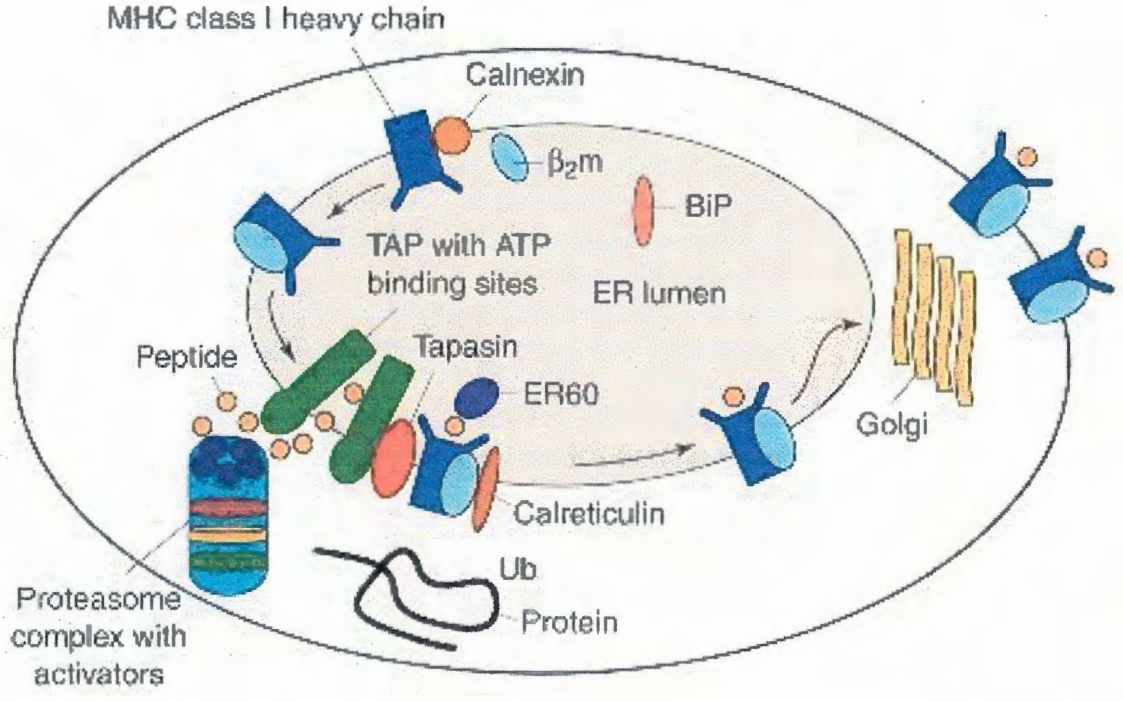


Fig. 1.5: The MHC class I antigen-processing pathway

Processing and presentation of peptides by MHC class I antigen processing machinery requires three major steps: a) the generation of antigenic peptides by the proteasome and other cytosolic proteases; b) peptide transport mediated by the TAP dimer from the cytosol into the ER; and c) the assembly of peptides with HLA class I molecules, which is assisted by various chaperones, such as calnexin, calreticulin, ER60 and tapasin. The trimeric complex comprising HLA class I H chains, β_2m and peptide is then transported through the Golgi directly to the cell surface for presentation to $CD8^+$ T cells (from Seliger et al.,2000).

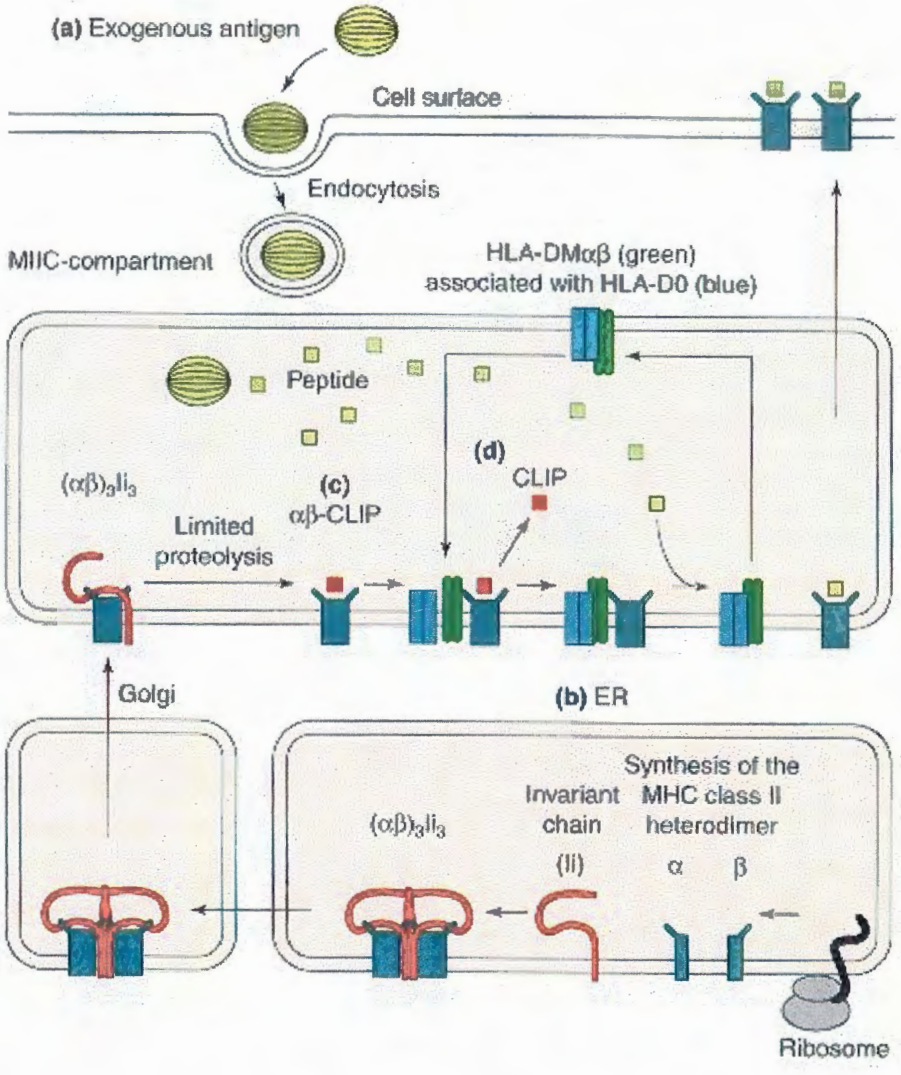


Fig. 1.6: The MHC class II antigen-processing pathway

MHC class II molecules present peptides derived from exogenous antigens internalized in the endocytic pathway (a). HLA class II heterodimers assemble in the ER (b) with the invariant chains (Ii) to form nonameric α/β-Ii complexes [$(\alpha\beta)_3Ii_3$], which are targeted to MHC class II compartments (MIIC) in the endocytic pathway. The HLA class II-associated Ii is degradated in distinct steps, leaving class II-associated Ii peptide (CLIP) within the HLA class II binding groove (c). CLIP can then be exchanged for antigenic peptides and this exchange is catalysed by HLA-DM molecules (d). The HLA-DM-dependent peptide loading is regulated by HLA-DO molecules. Peptide-loaded HLA class II molecules are then transported to the cell surface for presentation to $CD4^+$ T cells (from Seliger et al., 2000).

MHC class II molecule is formed by polypeptides α and β, similar in size and both anchored in the surface membrane at their carboxy termini; each class I molecule, by contrast, consists of a structurally distinct α chain associated with a second shorter polypeptide, called β_2-microglobulin. It is a polymorphic antigenic system initially recognised in transplantation experiments in tumour-bearing mice. These antigens, designated as HLA, are highly polymorphic membrane glycoproteins. The genetically inherited HLA alleles of an individual will determine the nature of the peptides capable of binding to these molecules. Thus, no immune response will occur against proteins which lack binding motifs for the HLA molecules of the host.

The level of expression of the HLA molecules by individual tumour cells is therefore important in determining the outcome of an immune response. Recently, a correlation between the level of expression of MHC on tumour cells and the presence of T cells in tumour tissue was observed. In a cervical carcinoma a decreased expression of monomorphic HLA class I expression correlated with a significant decrease of CD8+ T cells within tumour cell areas (Hilders et al., 1993).

For an effective elimination of tumour cells by cells of the immune system intercellular contact between the target and effector cell is required. These contacts involve, besides structures such as HLA or antigens for target cell recognition, structures for cell-cell binding (adhesion molecules) and for activation of immune cells (accessory molecules). One of the important interactions between effector cell and target cell is mediated by contact between LFA-1 and ICAM-1 (Melero et al., 1993). Tumour cells with relatively low expression of ICAM-1 are less susceptible to lysis by most effector cells (Braakman et al., 1990).

An important costimulatory signal is represented by the activation of CD28 receptor on the T cell by interaction with the B7 family of accessory molecules, expressed on APC

cells. This interaction is required for activation of lymphocytes and its proliferation, leading to clonal expansion (June et al., 1994).

1.2.3.2 (3) Th1 and Th2 paradigm

T helper cells serve an important function in the regulation of the immune response by the production of a variety of cytokines with autocrine and paracrine effects.

Many murine $CD4^+$ T cell clones secrete either of two profiles of cytokines which is reflected in the functions of these subsets (Paul et al., 1994; Mosmann et al., 1996). Th1 cells produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin, that enhance the development of cell-mediated inflammatory responses, including cytotoxic functions. Thus, a Th1-type reaction might be expected to favour an immune reaction against tumour cells. In contrast, Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, that promote an antibody response. Such a reaction would not be expected to promote a cytotoxic response against tumour cells, although antibodies may mediate an anti-tumour effect by sensitising tumour cells to macrophage attack.

One of the main factors that determine whether a Th1 or Th2 response develops is the nature of the cytokines themselves. A Th1 response is stimulated by IFN-γ and IL-12, while IL-4 favours a Th2 response. In addition, IFN-γ represses a Th2 response while IL-4 inhibits a Th1 reaction. Therefore, once a response starts proceeding in one pattern, it receives positive feedback along the same direction. However, early in the response, T cells are probably dependent on cytokines produced by other cells. For example, macrophages and dendritic cells produce IL-12, and NK cells produce IFN-γ when stimulated by IL-12, thus contributing to a Th1 response. In contrast, a Th2 reaction is favoured by mast cells and basophils, which secrete IL-4 (Fig. 1.7).

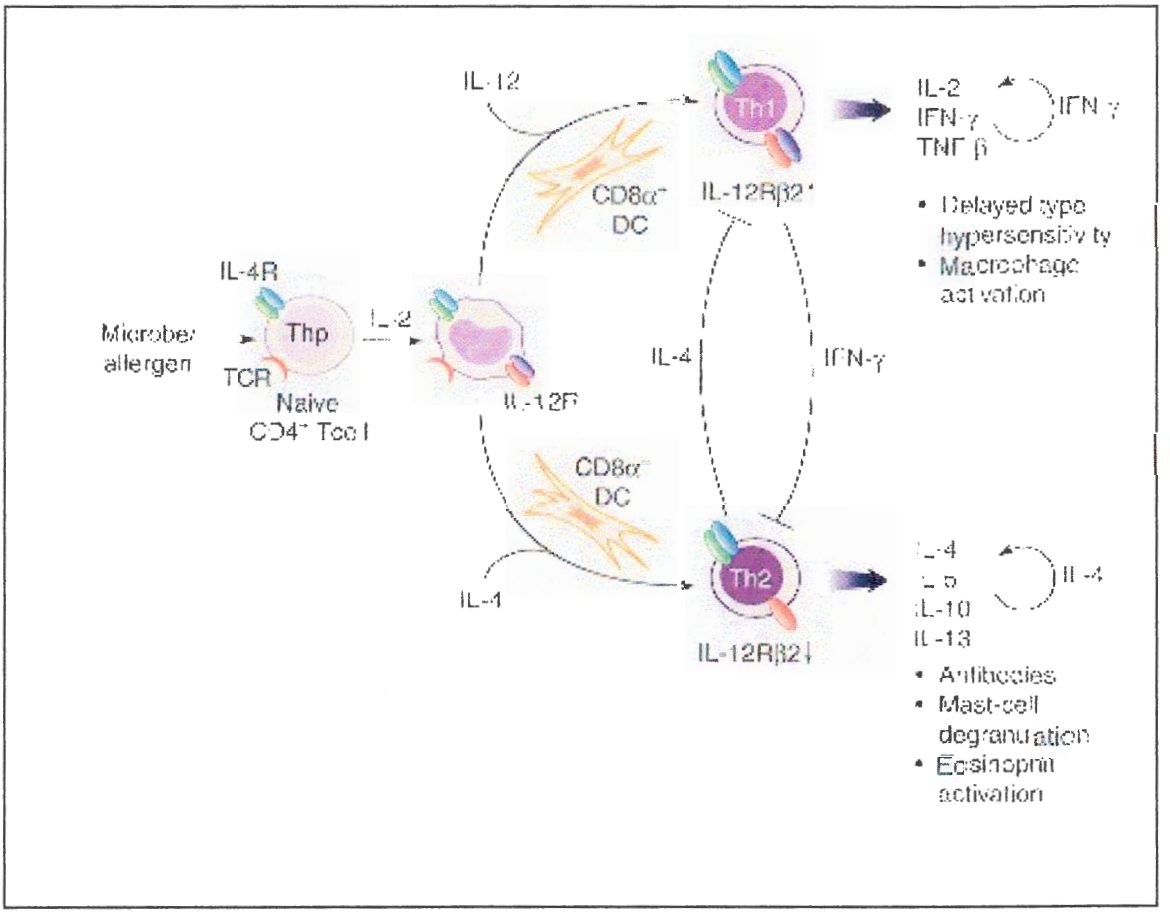


Fig. 1.7: Overview of Th cell differentiation

A naive $CD4^+$ T cell is activated via the TCR when it encounters antigen (for example, derived from a microbe or allergen) presented by an antigen presenting cell. Once activated, the Th cell starts to proliferate and secrete IL-2, and expresses the IL-12 receptor (IL-12R). On encountering IL-12 secreted by macrophages and/or on contact with $CD8\alpha^+$ dendritic cells (DCs), a Th1 differentiation program is initiated: the IL-12Rβ2 chain is upregulated in the developing Th1 cell and ligation of the IL-12R by IL-12 leads to activation of a specific transcription factor (Stat4) which initiates the Th1 differentiation process. IL-4 produced intrinsically by the T helper precursor and extrinsically by several cell types as well as contact with $CD8\alpha^-$ DCs induces differentiation into the Th2 subset: downregulation of IL-12Rβ2 expression and ligation of the IL-4R (expressed on the naive $CD4^+$ T-cell surface) by IL-4 activates a specific transcription factor (Stat6) which initiates the Th2 differentiation process (from Rengarajan et al., 2000).

The dose of the antigen also represents a determining factor of Th1/Th2 polarisation. With few exceptions, low antigen concentrations and low dose infections tend preferentially to induce Th1 responses, whereas high doses induce Th2 development (Bretscher et al., 1992; Hosken et al., 1995).

The mechanism underlying these effects of antigen dose is not well understood. It is possible that, at low doses of antigen, the principal antigen-presenting cells (APCs) are dendritic cells, or macrophages if the antigen is a particulate microbe. Both dendritic cells and macrophages produce IL-12 and tilt the balance of the specific T-cell response towards Th1 differentiation. Conversely, when the antigen concentration is high, it may be presented by APCs that do not secrete IL-12, thus favouring Th2 development. It is also possible that high concentrations of antigen lead to repeated T cell stimulation, thus increasing IL-4 production and Th2 development, or induce a state of immunological tolerance, which often preferentially shuts off Th1 cells (De Wit et al., 1992).

1.2.3.3 Natural killer (NK) cells

NK cells are a subset of lymphocytes, characterised morphologically as large granular lymphocytes, which are phenotypically CD3-negative, TCR ($\alpha\beta$ and $\gamma\delta$)-negative and CD16 positive (Abbas et al.).

A role for NK cells in tumour immunity *in vivo* is suggested by indirect evidence: experimentally induced and transplanted tumours grow more rapidly in NK-deficient animals (Talmadge et al., 1980; Karre et al., 1980).

NK cells can kill a wide range of tumour targets *in vitro*. The mechanism by which NK cells preferentially recognise and lyse transformed, rather than normal, targets is not well defined. In marked contrast to killing by CTLs, whose ability to recognise targets depends on antigen presentation by MHC class I molecules, natural killing is inhibited by MHC class I molecules and is enhanced by their absence. NK cells recognise but

receive an off-signal from class I molecules and thus preferentially lyse target cells with diminished expression of self-class I MHC molecules.

Studies of human NK cells have led to the identification of a set of NK cell inhibitory molecules: the killer cell inhibitory receptor (KIR) family. KIR molecules are members of the immunoglobulin supergene family and are expressed as monomers on NK cell membrane, oriented with their amino termini extracellularly. These molecules interact with MHC class I molecules and, as a result of this interaction, inhibit natural killing. Individual KIR molecules, which appear to consist of at least 10 members of the family, differ in the particular class I molecules they recognise. Differential expression of KIR molecules may therefore generate subsets of NK cells with differing abilities to kill targets. The ability of NK cells to kill MHC class I^- cells clearly implies the existence of an "on signal" whenever NK cells interact with potential target cells. The surface receptor(s) responsible for NK-cell triggering in the process of natural cytotoxicity remained unknown until recently. These surface receptor(s), are collectively termed "natural cytotoxicity receptor" (NCRs) (Abai et al., 1999). Moreover, some data suggest the existence of other molecules which function as co-receptors in human NK cell triggering. A suitable candidate for this function is the 2B4 molecule (Moretta et al., 2000) (Fig. 1.8).

Cytolysis by NK cells is mediated by the release of a cytotoxic factor(s) and the use of perforins to puncture holes in the target cell membrane.

The cytotoxic activity of NK cells can be augmented both *in vitro* and *in vivo* with the lymphokines interleukin-2 (IL-2) and interferon, and thus NK activity can be amplified by immune T cell response. Therefore, NK cells may represent a first line of host defense against the growth of transformed cells at both the primary and metastatic sites, as well as providing an effector mechanism recruited by T cells to supplement specific antitumour response.

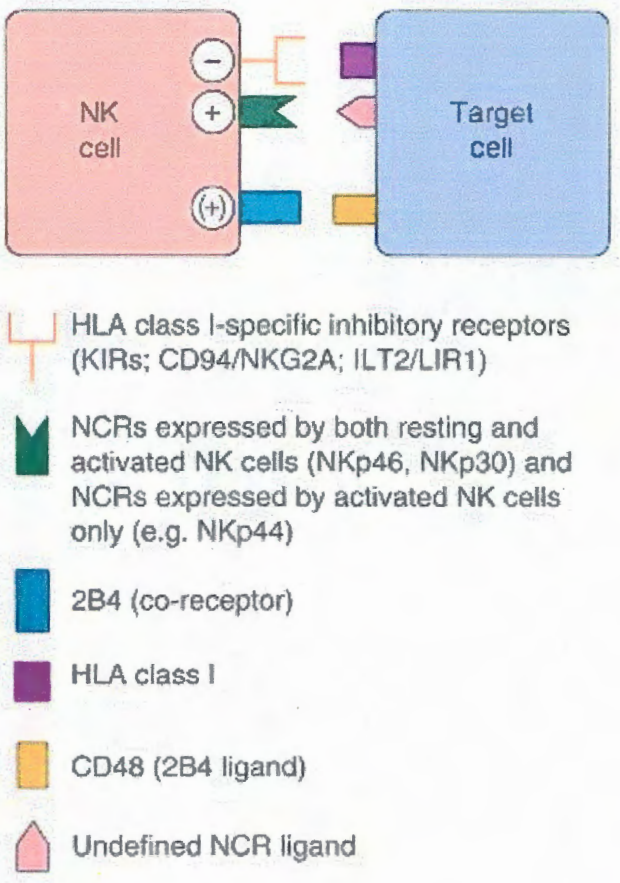


Fig. 1.8: Model of the surface receptors involved in natural killer (NK)-cell activation or inactivation

Upon interaction with potential target cells, activating receptors expressed at the NK-cell surface, including constitutively expressed (NKp46 and NKp30) and inducible (NKp44) natural cytotoxicity receptors (NCRs), interact with their ligands on target cells. In the absence of concomitant inhibitory signals (in the case of HLA class I$^-$ cells), this leads to NK-cell triggering and target cell lysis. In the case of normal cells expressing HLA class I molecules, the interaction between inhibitory receptors and HLA class I molecules delivers inhibitory signals that overcome NK-cell triggering, thus preventing target-cell lysis. The activation function of the 2B4 coreceptor is dependent upon the simultaneous engagement of the NCRs (from Moretta et al., 2000).

An interesting subpopulation of NK cells has been defined over the past 10 years. This population, defined NK1.1 $^{+}$ T cells, coexpresses an invariant T cell antigen receptor (TCR) α chain, encoded by the $V_{\alpha}14$ and $J_{\alpha}281$ gene segments, associated with polyclonal $V_{\beta}8$, $V_{\beta}7$ and $V_{\beta}2$ TCR β chains, and receptors of the NK lineage.

These cells have the unique potential to very rapidly secrete large amounts of cytokines, providing early help for effector cells and regulating the Th1 or Th2 differentiation of some immune responses. NK1.1 $^{+}$ T cells recognise the products of the conserved family of MHC class I-like CD1 genes, which represent a family of non-polymorphic molecules able to bind and present lipid-containing antigens derived from intracellular pathogens.

NK1.1 T cells were found to be an essential target of IL-12-mediated tumour rejection. Indeed, interleukin-12-induced rejection of tumours were not observed in NK1.1 cell-deficient mice, characterised by a specific deletion of Ja281 gene segment and thus lacking of NK1.1 subpopulation (Cui et al., 1997).

1.2.3.4 Macrophages

Macrophages are potentially important in anti-tumour immunity as antigen-presenting cells to initiate the immune response and as potential effector cells to mediate tumour lysis. Resting macrophages are not cytolytic to tumour cells *in vitro* but can become cytolytic if activated with macrophage-activating factors (MAF). MAF are commonly secreted by T cells following antigen-specific stimulation; T-cell lymphokines with MAF activity include interferon gamma, TNF, IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The mechanisms by which macrophages recognise tumour cells and mediate lysis are not defined, but activated macrophages bind to and lyse transformed cells in marked preference to normal cells. Several distint lytic mechanism may be operative, depending on the MAF responsible for activating the

macrophages. These include intercellular transfer of lysosomal products, superoxide production, release of neutral proteinases and secretion of the monokine TNF (Abbas et al.). Macrophages express Fcγ receptors; thus, a further mechanism may be represented by their targeting to tumour cells coated with antibody.

1.1.4 MECHANISMS OF TUMOUR ESCAPE

Malignant melanoma has been the human tumour system most extensively studied, because of the greater availability of TAA-specific T cells that can be isolated from patients with this disease. Several CTL-defined melanoma-associated antigens (MAA) have been identified. Some of the identified human MAA have already undergone testing in patients and a large amount of data has been rapidly accumulated on the immune and clinical responses these MAA elicit (Nestle et al., 1998; Rosenberg et al., 1998). These studies have shown that in the majority of patients immunisation with MAA does not yield the clinical responses predicted by murine models. Furthermore, progression or recurrence of disease has been observed in spite of the induction or persistence of MAA-specific CTL response. The utilisation of defined MAA or peptides, derived from their sequence, for vaccination of patients with advanced melanoma has yielded an opportunity to analyse the dynamics of *in vivo* interactions between the host immune system and tumour cells in human and to analyse in detail the molecular mechanism utilised by tumour cells to escape from CTL recognition.

Many potential mechanisms permitting escape from immune destruction have been identified.

1.1.4.1 Inadequacy of tumour cells as targets

The recognition of tumour cells by CTL requires a sufficient presentation to TCR of tumour associated antigen (TAA) derived peptides in association with the restricting HLA class I allele. Defects in expression of either TAA or HLA class I molecules, as well as those molecules involved in generation of peptides from TAA and transport through the tumour cell, can provide tumour cells with an escape mechanism from CTL recognition.

1.1.4.1 (1) TAA loss or down-regulation

Alteration in TAA expression is one of the mechanisms by which tumour cells may escape CTL recognition *in vivo*. Changes in TAA expression range from down-modulation to total loss.

Using the murine mastocytoma P815 tumour model, Biddison and Palmer (Biddison et al., 1977; Uyttenhove et al., 1983) presented the first evidence that tumour escape may be due to the emergence of stable antigen-loss variants. Tumour cells of the subline P815-Y that were collected from the peritoneal cavity of tumour bearing animals a few days after the partial rejection phase were less sensitive to CTL lysis than the original tumour cells. The molecular basis for this finding subsequently was elucidated by Boon and his colleagues (Van den Eynde et al., 1991a; Boon et al., 1992; Lethe et al., 1992). These authors identified the gene P1A, silent in normal tissues with the exception of testes, which encodes the mastocytoma's tumour rejection antigen. This antigen is composed of two distinct CTL epitopes. Because of deletions within the P1A gene, both epitopes are undetectable in P815 mastocytoma cells that escape tumour rejection *in vivo*. This finding provided convincing evidence that tumour cells can escape from CTL recognition because of the development of variants that no longer express the rejection antigen(s) recognised by TAA-specific CTL.

Most knowledge about alterations in antigen expression in human tumours comes from studies performed with melanoma cells, which demonstrated an heterogeneity in both CTL-defined and antibody-defined MAA expression. For example, among members of the MAGE family, studies utilising reverse transcriptase polymerase chain reaction (RT-PCR) showed that MAGE-1 was not expressed in a significant proportion of melanoma lesions, while MAGE-3 had a wide distribution in melanoma and in other malignancies (Van der Bruggen et al., 1991). The differences in degree of expression among members of the MAGE family and other cancer testis antigens appear to reflect

different susceptibility to a genome-wide demethylation process associated with tumour progression (de Smet et al., 1996). Furthermore, studies using mAb specific for gp100, Mart-1/MelanA and tyrosinase have revealed that most primary melanoma lesions express these tumour differentiation antigens (De Vries et al., 1997; Busam et al., 1998), while metastases are quite heterogenous in their expression (Scheibenbogen et al., 1996; De Vries et al., 1997; Cormier et al., 1998; Riker et al., 1999). In particular, gp100 and Mart-1/MelanA have not been detected in about 10-20% of metastatic lesions, whereas tyrosinase appears to be less frequently lost (Chen et al., 1995; Cormier et al., 1998).

1.1.4.1 (2) HLA class I antigen loss or down-regulation

Selective loss of MHC class I alleles was first described in several mouse tumours, including the TL leukaemia cell line, the T10 sarcoma, the 3LL Lewis lung carcinoma and the B16 melanoma. It was soon realised that this phenotypic alteration may permit tumour cells to avoid and to survive attack by the immune system. This notion was supported by the enhanced growth of mouse tumour cells which had down-regulated MHC class I antigen expression following transfection with antisense DNA (Hui, 1989) and by the loss of tumourigenicity of aggressive MHC class I–negative mouse tumour cells following transfection with MHC class I genes (Hui et al., 1984a).

HLA class I antigens are lost or down-regulated in many types of human tumours. The most common types of solid tumours analysed for HLA class I antigen expression include melanoma and carcinoma of the breast, cervix, colon, head and neck squamous cell, kidney and prostate.

The altered HLA class I expression is summarised from these studies as loss or down-regulation of monomorphic determinants or selective down-regulation of one or more

allospecificity(ies). Several distinct molecular mechanism that underlie the various phenotypes have been identified.

Total HLA class I antigen loss in tumour cells is caused by mutations in the β_2m gene that result in loss of functional $\beta_2$microglobulin expression. Mutations in the β_2m gene have been described in colon and lung carcinoma and in malignant melanoma (D'Urso et al., 1991; Wang et al., 1993; Bicknell et al., 1994; Chen et al., 1996; Hicklin et al., 1997; Hicklin et al., 1998; Benitez et al., 1998).

In contrast to total loss, HLA class I antigen down-regulation on tumour cells can reflect a defect in the regulatory mechanism responsible for the expression of HLA class I heavy chains. For example, tumour cell lines with HLA class I antigen down-regulation were shown to have altered binding of regulatory factors to HLA class I heavy chain gene enhancer elements (Henseling et al., 1990; Blanchet et al., 1991; Blanchet et al., 1992).

Abnormalities in HLA class I antigens may selectively affect only some of the HLA class I allospecificities encoded in malignant cells. Loss of an HLA class I haplotype has been described in malignant melanoma and pancreatic carcinoma (Marincola et al., 1994; Torres et al., 1996), and a selective down-regulation of a gene product of the HLA-A or HLA-B locus has been found frequently in tumour cell lines (Marincola et al., 1994; Hicklin et al., 1998) and in surgically removed lesions (Kim et al., 1996a).

1.1.4.1 (3) Defects in HLA class I-dependent antigen processing

HLA class I antigen down-regulation frequently is associated with defects in antigen processing. Correct assembly of HLA class I molecules and efficient presentation of antigenic peptides is dependent on the generation of peptides by the proteasome complex and the transport of these peptides into the endoplasmic reticulum, where they are assembled with HLA class I heavy chains and β_2-microglobulin. Tumour cells may

alter expression of components of the HLA class I antigen processing pathway leading to abnormal processing and presentation of TAA.

Several studies have demonstrated abnormal expression of the proteasome subunits LMP2 and LMP7 and/or the transporter subunits TAP1 and TAP2 in cell lines of hepatocellular, lung, prostate and renal carcinomas, lymphoma, malignant melanoma and neuroblastoma (Seigler et al., 1971; Ferrone et al., 1995; Rowe et al., 1995; Sanda et al., 1995, Alpan et al., 1996; Kurokohchi et al., 1996; Seliger et al., 1996; Singal et al.,1996; Johnsen et al., 1998; White, 1998). Simultaneous down-regulation of multiple components has been detected in several cell lines. The LMP and/or TAP gene transfection of these cell lines restores HLA class I presentation of antigens and tumour cell recognition by antigen-specific CTL.

Little information is available about the molecular basis of LMP and/or TAP down-regulation in malignant cells. Synchronous loss of LMP and TAP subunits in some tumour cell lines suggests that various mechanisms of gene regulation are defective in these cells; in support of this hypothesis, this phenotype can be corrected by treatment with IFN-γ (Restifo et al., 1993; Seliger et al., 1996; Johnsen et al., 1998; White, 1998).

To date only one study has described a genetic mutation in tumour cells involving an antigen processing molecule, represented by a point mutation in the TAP1 gene in a lung carcinoma cell line (Chen et al., 1996). This mutation introduces a premature stop codon which results in a nonfunctional TAP1 protein (Chen et al., 1996).

1.1.4.2 Potential role of HLA class I polymorphism in tumour cell escape

Many melanoma-associated antigens (MAA) contain multiple peptide sequences that can function as T cell epitopes in the context of the same or different HLA class I alleles. Bulk TIL populations include subpopulations of T cells specific for more than one MAA. Thus, the host immune response against cancer cells potentially is broad.

Contrasting with this potential wealth of T cell/tumour cell interactions is, however, the practical observation that most often MAA immunodominance is restricted to few HLA class I alleles and TIL recognise predominantly one MAA epitope for each tumour. This phenomenon, which is referred to as epitope immunodominance, reflects discrepancies between the number of peptides within an antigenic protein that could bind to a particular HLA class I allele and the number of epitopes actually recognised in a CTL response to that protein (Kim et al., 1998b).

Most MAA preferentially function as immunogens in association with particular restriction elements. For example, clonal analysis of MART-1/MelanA-specific CTL showed that MART-1/MelanA immunodominance is strongly restricted to the epitope resulting from the MART-1_{27-35} peptide in association specifically with the HLA-A0201 allele (Kawakami et al., 1994b; Rivoltini et al., 1995). Thus, autologous induction of MART-1/MelanA-specific CTL by whole antigen processing and presentation is restricted to a unique HLA class I allele/ligand combination and is excluded by minimal changes in HLA class I allele structure.

Thus, this strong restriction to a unique HLA class I allele/ligand combination may have important implications in relation to escape mechanisms. Indeed, the loss of the specific combination, if due to a limited defect in only one of the two molecules could result in the lack of presentation of the tumour antigen.

1.1.4.3 Inhibitory signals provided by the tumour microenvironment

Other mechanisms, which do not involve the direct interaction of T cells with the MHC class I-restricted tumour associated antigen, can independently affect tumour-host interactions and represent alternative means of tumour escape.

1.1.4.3 (1) Immunoregulatory cytokines

Within the microenvironment, host defenses against the tumour are controlled by immunological mediators, particularly cytokines.

Secreted cytokines can have a stimulatory or an inhibitory role in immune response. Tumour cells, as well as normal cells, indeed, can spontaneously release in the tumour microenvironment immunosuppressive cytokines, that inhibit host immune function (Chouaib et al., 1997), as well as stimulatory factors, that make tumour cells more susceptible to recognition.

Several lines of evidence have demonstrated the presence of different cytokines in the tumour microenvironment.

For example, recently, IL-6 has been shown to be expressed specifically in the tumour microenvironment in 83% of patients with colorectal carcinoma (Piancatelli et al., 1999); this cytokine, which is known to have multiple effects on T cell function may play a significant role in modulating T cell reactivity at the tumour site.

IL-10 has pleiotropic effects on T-cell functions. These include suppression of granulocyte macrophage colony stimulating factor, IFN-γ and IL-2 production by T helper cells, inhibition of T cell proliferation, downregulation of expression of adhesion molecules and HLA class I and class II antigens. Although immune cells represent the major source of IL-10, this cytokine can also be secreted by melanoma cell lines and can be found in tissue samples from patients with metastatic melanoma (Dummer et al., 1996).

TGF-β, which has a predominant role as suppressor of CTL function, has been demonstrated to be secreted by different tumour cell lines (Dummer et al., 1996; Gorsch et al., 1992).

In situ expression of TGF-β is common in tumours and correlates with progression of melanoma (Schmid et al., 1995; Van Belle et al., 1996; Moretti et al., 1997) and other

skin tumours (Schmid et al., 1996). Particularly TGF-β2 and TGF-β3 are expressed in melanoma lesions but are not detectable in melanocytes. Furthermore, the level of TGF-β tends to increase with tumour progression because it is lower in thin primary melanoma lesions than in thick primary melanoma lesions and in metastases (Moretti et al., 1997). It has been proposed that the presence of TGF-β in situ is due to the paracrine secretion of this cytokine by tumour cells, as occurs in cell lines from melanoma metastases (Rodeck et al., 1991).

Cytokines present in the tumour microenvironment may modulate local interactions between CTL and tumour cells via many different mechanisms. For example, cytokines can alter the expression of adhesion molecules on the peritumoural endothelium (Yoong et al., 1998) and on target tumour cells (Vanky et al., 1990; Cao et al., 1997; Lefor et al., 1998). The presence of adhesion molecules facilitates trafficking of T cells to the tumour site and reinforces the physical interaction of T cells with tumour cells. For example, ICAM-1, which is known to reinforce CTL/tumour interactions, can be induced or enhanced in normal and malignant cells *in vitro* and *in vivo* by IFN-γ, interleukin (IL)-10 and tumour necrosis factor-α (TNF-α) (Temponi et al., 1988; Maio et al., 1989; Maio et al., 1989; Mortarini et al., 1990; Yue et al., 1997).

Expression of adhesion molecules, such as ELAM-1, ICAM-1 and VCAM-1, may influence tumour progression (Johnson, 1991; Dyer, 1999; Maurer et al., 1998; Terol et al., 1998) and various malignant cell lines or tissues, including melanoma, have been demonstrated to have heterogeneous and often decreased expression of adhesion molecules (Mortarini et al., 1990; Braendstrup et al., 1996; Budinsky et al., 1997).

1.1.4.3 (2) Surface expression of apoptotic signals

Cancer cells are frequently resistant to apoptosis mediated through Fas. It has been reported that high levels of Fas ligand (FasL) are expressed in a high percentage of melanoma cell lines and surgically removed melanoma tumours. Furthermore, high levels of FasL were also reported to be present in sera from patients with melanoma (Hahne et al., 1996).

These findings suggested a novel mechanism utilised by tumour cells to escape from T cell recognition. Through interaction with Fas on the surface of TIL, FasL could counterattack and extinguish TAA-specific CTL at the tumour site. This model provides a mechanism for the lack of tumour regression in spite of the presence of TAA-specific CTL in malignant lesions.

Besides melanoma, detection of FasL in a number of cell lines and a variety of surgically removed malignant lesions has been reported. The tumours include astrocytoma (Saas et al., 1997), glioblastoma (Gratas et al., 1997), esophageal (Bennett et al., 1998), lung (Niehans et al., 1997), hepatocellular (Strand et al., 1996) and colon (O'Connell et al., 1996; Shiraki et al., 1997) carcinoma.

In addition to the expression of high level of FasL, other mechanisms have been demonstrated to be responsible for cancer cells resistance to apoptosis. This might be a result of downregulation of Fas or the release of soluble Fas, or abnormalities in the level of several proteins involved in the signal transduction cascade. Moreover, there is some evidence that some oncogene and tumour suppressor gene mutations, commonly found in tumours, could potentially impair Fas signalling (O'Connell et al., 1999) (Fig. 1.9).

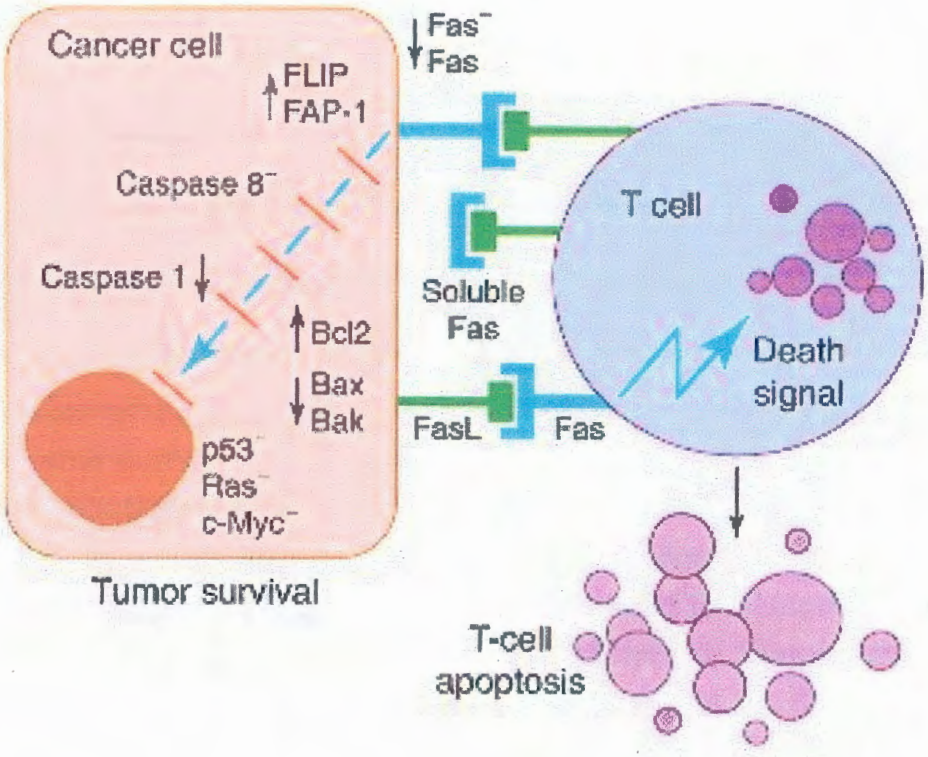


Fig. 1.9: Mechanisms of resistance to apoptosis

Cancer cells are frequently resistant to apoptosis mediated through Fas. This might be a result of downregulation of Fas or release of soluble Fas, or abnormalities in the level of several proteins involved in the signal transduction cascade (downregulation of caspase 1, Bax or Bak, and upregulation of FLIP, FAP-1 or Bcl2). In addition, mutations have been identified in some components of the pathway, including Fas itself and caspase 8. There is evidence that some oncogene and tumour suppressor gene mutations, commonly found in tumours, could potentially impair Fas signaling (p53 and Ras) or cooperate to Fas resistance (c-Myc). Many cancer cells also express FasL and can therefore counterattack and kill Fas-sensitive tumour-infiltrating lymphocytes (TILs) (from O'Connel et al., 1999).

1.3 CANCER IMMUNOTHERAPY APPROACHES

It has been more than 100 years since the first reported attempts to activate a patient's immune system to eradicate developing cancers. In the 1890s, William Coley began to treat cancer patients with bacterial extract's (Coley' toxins) to activate general systemic immunity, a portion of which might be directed against the tumour.

The molecular understanding of immune recognition and the molecular identification of tumour specific antigens provided opportunities to create immunotherapy approaches with much greater potency and specificity for tumour cells and diminished toxicity for normal tissues (Fig. 1.10).

Three basic approaches have been used to treat patients with cancer using immunotherapy:

- passive or adoptive immunotherapy, in which anti-tumour immune response is augmented by infusion in patient of tumour-reactive immune cells or antibodies;
- therapy with cytokines, in which immune response is amplified by administration of one or more cytokines;
- active immunotherapy or vaccination, in which the immune system is stimulated to respond to tumour antigens, by administration of antigens, presented in variable forms, as vaccines.

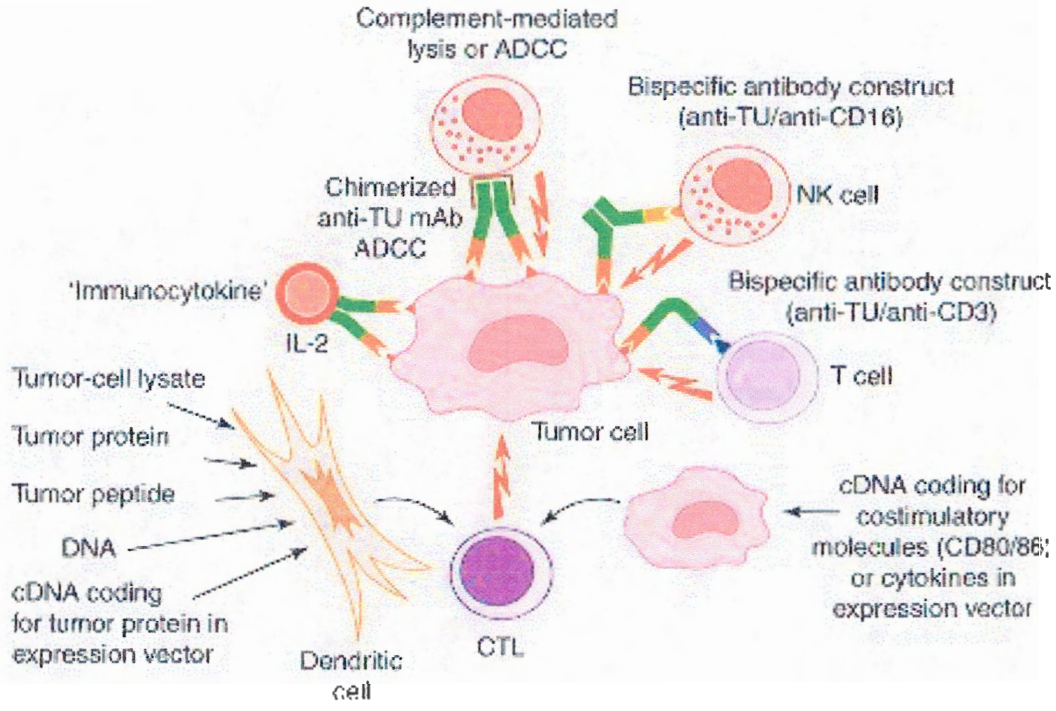


Fig 1.10: Overview of current strategies in experimental immunotherapy of tumours

Tumour-specific mAbs can mediate cytolysis either by engaging NK cells via Fc receptors (ADCC) or by complement activation. Bispecific antibody construct are designed to bring immune effector cells into contact with tumour cells and to simultaneously stimulate their cytotoxic activity. Recombinant fusion products of antitumour antibodies and cytokines can concentrate immune effector functions at the tumour site. CTLs can be activated against tumour antigens by tumour cells rendered immunogenic by expression of either costimulatory molecules or cytokines. Effective stimulation of tumour-specific CTLs is achieved by dendritic cells loaded with peptides or exposed to tumour-cell lysates, tumour proteins, DNA, or transfected with cDNA coding for tumour protein in an expression vector (from Ockert et al., 1999).

1.3.1 PASSIVE OR ADOPTIVE IMMUNOTHERAPY

1.3.1.1 Cellular adoptive immunotherapy

Cellular adoptive immunotherapy of malignancies involves the passive transfer of antitumour-reactive cells into a host in order to mediate tumour regression. T cell populations that recognise tumour can be derived from cancer patients expanded *in vitro* to large numbers and used for the adoptive immunotherapy of cancer.

The *in vitro* expansion and subsequent infusion of tumour-specific T cells into tumour-bearing hosts has proven efficacious in a variety of murine tumour models (Cheever et al., 1986; Greenberg et al., 1985; Greenberg, 1991). Both CD4- and CD8-positive T cells have activity, apparently independent of the MHC status of the tumour, suggesting that multiple antitumour effector mechanisms can be recruited. In addition to rejecting the neoplasm, these animals developed a long lasting systemic immunity (Fass et al., 1972; Colombo et al., 1985).

However, clinical studies in patients using cellular adoptive immunotherapy demonstrated variable results and limited success and its potential efficacy is still under debate.

Tumour-reactive T cells have been generated from several sources including peripheral blood, the site of growing tumour and lymph nodes draining tumours.

The first clinical protocols describing the use of cellular adoptive immunotherapy in patients with renal cell carcinoma and melanomas reported that more than 30% of patients with advanced melanoma had an objective clinical response (Rosenberg et al., 1987). These results were obtained with the use of peripheral blood lymphocytes (PBL), activated *in vitro* in the presence of high doses of recombinant interleukin-2. These cells, named lymphokine activated killer (LAK) cells, have the capability of lysing not only autologous, but also allogeneic cancer cells. The clinical results obtained were

interesting but the severe toxicity of the approach, associated with a number of treatment-related deaths, limited its application in clinical trials.

Tumour infiltrating lymphocytes subsequently were used. These cells are concentrated *in vivo* at the tumour site and can be isolated and expanded in culture in the presence of recombinant IL-2. At least a fraction of TIL was shown to kill human tumour cells in an HLA class I-restricted fashion, suggesting specific recognition of tumour antigens. These cells were used in adoptive cellular immunotherapy of advanced melanoma patients with promising clinical results (Rosenberg et al., 1988). Indeed, a variable percentage of patients (up to 40% in some studies) had objective clinical responses with the use of intravenous infusion of several billions (100-700) of *in vitro*-expanded TIL, in combination with high doses of rIL-2. However, further clinical studies using lower doses of IL-2 to reduce the very severe toxicities observed in the original trials, reported lower percentage of responses (Dillman et al., 1991; Arienti et al., 1993; Goedegebuure et al., 1995).

Methods to genetically modified TIL to enhance their antitumour abilities and optimise adoptive immunotherapy have been developed by gene transfer techniques (see chapter 1.4).

1.3.1.2 Antibody therapy

Monoclonal antibodies (MoAb) developed against tumour associated antigens have been tested in a variety of forms in different clinical settings.

Antibodies can activate immune effector functions. Antibody that binds to the target antigen can mediate cell killing by complement fixation, opsonization or antibody-dependent cell-mediated cytotoxicity.

In the last few years, the availability of recombinant engineering techniques to manipulate the structures of antibody-based proteins has revolutionised their use in

cancer therapy. In order to reduce immunogenicity, humanised antibodies have been constructed by engineering chimeric antibodies with a human backbone (Winter et al., 1991) or by production from transgenic mice (Jakobovits et al., 1995). Chemically digested and recombinantly prepared antibody fragments have been created to improve tumour penetration and rapid systemic clearance (Adams et al., 1998).

Multiple antibody-based strategies have been developed and used in therapy of cancer.

1.3.1.2 (1) Unconjugated antibodies

The effectiveness of unconjugated monoclonal antibody depends on the ability of these molecules to accumulate at tumour sites and then stimulate the biological response. Several clinical trials have been conducted using unconjugated antibodies and their derivatives.

In a randomised trial, 189 colorectal cancer patients, who had undergone curative resection for Duke's C cancer, received either monclonal antibody to the tumour-associated antigen 17-1A, a 34-KDa glycoprotein of the cell membrane of epithelial cells, or were assigned to an observation arm (Riethmüller et al., 1994). At a median follow-up of 5 years, the mortality rate and recurrence were reduced by 30% and by 27%, respectively, in the treatment group compared to controls. By 7 years of follow-up, the survival benefits were maintained (Riethmüller et al., 1994).

Encouraging results have also been demonstrated with antibody-based therapy in leukaemias and lymphomas (Kaminski et al., 1996). A chimeric monoclonal antibody to CD20 recently was licensed for use in relapsed low grade or follicular non-Hodgkins B cell lymphoma (Maloney et al., 1998).

In patients with metastatic breast cancer, who had received extensive prior anti-cancer therapy, treatment with anti-p185 HER2, Herceptin, produced response rates comparable to third or fourth-line chemotherapy, but with minimal toxicity (Baselga et

al., 1996). The antibody binds to HER2/c-erbB2, a transmembrane tyrosine kinase receptor overexpressed in approximately 30% of primary breast cancers. A multicentre controlled trial of 469 women with HER2-positive metastatic breast cancer was randomised so the women received doxorubicin and cyclophosphamide or paclitaxel with or without Herceptin. At a median follow-up of 25 months, a significant survival benefit for women receiving concurrent Herceptin was observed (Slamon et al., 1998).

1.3.1.2 (2) Conjugated antibodies

In contrast to immunologically oriented strategies, antibodies used in immunoconjugates are designed to provide targeting specificity to cytotoxic moieties. Monoclonal antibodies have been conjugated to catalytic toxins, chemotherapeutic agents and radionuclides.

Several plant and bacterial catalytic toxins have been used (Fitzgerald et al., 1989; O'Toole et al., 1998). These toxins require internalisation and intracellular processing to exert their effect by inhibiting protein synthesis. The internalisation is mediated by a cell-binding chain, which, in the construction of immunotoxins, is substituted by specific anti-tumour monoclonal antibody, able to target the toxin to the tumour cell.
Clinical trials have been performed using immunotoxins in patients with breast cancer, ovarian cancer, colorectal cancer, melanoma and lymphoproliferative disorders. In a study on 14 patients with B-cell lymphoma, five clinical responses were observed after treatment with an anti-CD22, a B cell marker, monoclonal antibody conjugated to ricin A chain (Vitetta et al., 1991).

Monoclonal antibodies are also used to deliver conventional chemotherapy agents to tumour sites. Preclinical results were obtained using the immunoconjugated BR96-

doxorubicin (Trail et al., 1993) in rodent models; however, a dose-limiting toxicity was observed in a series of phase I clinical trials.

In contrast to toxin- and drug-conjugates, radioimmunoconjugates can exert their toxic effect over several cell diameters from the radiation source and do not require internalisation for their activity. Stable labelling techniques have been developed to prevent radionuclides from dissociating *in vivo*, and improved linkers have been obtained for appropriate monoclonal antibody stability.

A radiolabelled conjugate of the anti-CD20 monoclonal antibody was used in clinical trials in highly pretreated patients with recurrent low-grade lymphoma. A complete response rate of greater than 80% and a 62% rate of progression-free survival at 2 years was observed (Wahl et al., 1998).

Novel immunoconjugates composed of antibodies and cytokines have been recently created. These molecules can establish cytokine/chemotactic gradients to encourage cytotoxic leukocyte accumulation and proliferation at tumour site (Sabzevari et al., 1994; LeBerthon et al., 1991).

1.3.1.2 (3) Bispecific antibodies

Bispecific antibodies are double-specific monoclonal antibodies, designed to activate a cellular immune response by cross-linking immune cells to tumour cells. The simultaneous recognition of a target antigen and of a cytotoxicity trigger on lymphocytes, mediated by the same antibody molecule, can induce the destruction of tumour cells by the effector cells (Segal et al., 1988). Such molecules can be prepared by chemical conjugation, hybridoma fusion or by creation of recombinant molecules.

Clinical trials have been conducted using bispecific antibodies targeting T cells via CD3. Intraperitoneal administration of a bispecific antibody directed against the α-folate receptor, which is overexpressed on 90% of ovarian carcinoma, and CD3 in combination with autologous lymphocytes and interleukin-2 induced a high proportion of clinical responses in patients with stage III ovarian carcinoma (Canevari et al., 1995). Bispecific antibodies targeting HER2/neu and the high-affinity Fcγ receptor (Valone et al., 1995), expressed by monocytes and activated neutrophils, or the low affinity Fcγ receptor (Weiner et al., 1995), expressed by natural killer cells and mature macrophages, exhibited some clinical activity in a phase I trial.

1.3.2 CYTOKINE THERAPY

Cytokines have been used to enhance MHC-restricted and unrestricted tumour cytotoxicity.

1.3.2.1 Interferons

Interferon-α (IFNα) is perhaps the most widely studied biologic response modifier in current use. By upregulating the expression of MHC class I and class II, as well as tumour-associated antigens, IFNα renders tumour cells more susceptible to destruction by T lymphocytes. It also stimulates the cytolytic activity of natural killer cells and cytotoxic T lymphocytes.

IFN-α has been used in the treatment of malignant melanoma patients. Early reports showed evidence of activity for IFNα in the treatment of metastatic disease (Nethersell et al., 1985). However, the overall response rates were no better than the most active cytotoxic drugs.

Clinical trials with high doses of IFNα in patients with high-risk node-positive stage III resected melanoma (Creagan et al., 1995) or low dose of IFNα in patients with intermediate-risk melanoma (Grob et al., 1998; Pehamberger et al., 1998) demonstrated a prolongation in relapse-free survival, but no benefit in overall survival from treatment. Recent evidence suggests a possible benefit for high-dose IFNα as an adjuvant therapy. In a randomised controlled trial, adjuvant therapy with IFNα significantly increased 5-year disease-free and overall survival in patients with high-risk resected malignant melanoma (Kirkwood et al., 1996), although toxicity from high-dose IFNα was observed.

Studies using IFNα have been also performed in renal cancer treatment. Patients with metastatic renal cancer showed benefit in survival by treatment with IFNα, but the benefit was achieved at the cost of substantial toxicity (MRC Collaborators, 1999). A study of patients with progressive metastatic disease suggested a benefit for combination therapy with IL-2 and IFNα (Negrier et al., 1998).

1.3.2.2 Interleukin-2

Interleukin-2 (IL-2) has virtually no direct effect on cancer cells and its anti-tumour activity *in vivo* is immunologically mediated. Physiologically, IL-2 serves as an important growth and activation factor for T lymphocytes. It also stimulates macrophages, NK cells and B lymphocytes and, at very high concentrations, IL-2 can induce NK cells and some T cells to become cytolytic against a variety of tumour cells in a non-specific and non-MHC-restricted manner.

The intravenous infusion of IL-2 combined with autologous lymphokine activated killer (LAK) cells resulted in objective responses in over 30% of melanoma patients (Rosenberg et al., 1987). Subsequent studies showed that LAK cells could be omitted from the treatment schedule without affecting the response rate (Law et al., 1995).

In a multicentre study, 14% of complete or partial responses lasting for a median of 23 months were observed in 255 patients with metastatic renal cancer treated with high-dose IL-2 (Fyfe et al., 1995; Fyfe et al., 1996). More recently, similar response rates were obtained with lower dose IL-2 given subcutaneously, with considerably less toxicity (Law et al., 1995).

1.3.2.3 Tumour necrosis factor

Tumour necrosis factor-α (TNF-α), a cytokine secreted by activated T lymphocytes and macrophages, has both direct and indirect antitumour effects. Although it is cytotoxic to some types of transformed cells *in vitro*, much of its effects are on the vascular endothelium, platelets, and the coagulation cascade, which lead to a rapid hemorrhagic necrosis of tumours, hence its name. TNF-α also potentiates the activation of CTL by its effects on T cells and tumour cell targets. Unfortunately, despite impressive activity in murine tumour models, the toxicity of systemic TNF-α at therapeutic doses has been prohibitive in humans (Mueller, 1998).

1.3.2.4 Interleukin-12

Interleukin-12 (IL-12) induces IFN-γ secretion, promotes growth of activated T and NK cells and induces commitment from the T helper 0 (Th0) to the Th1 phenotype.

A number of presentations addressed the potent antitumour effects of IL-12 in murine tumour models (Salgaller et al., 1998; Trinchieri, 1997). This activity appears to be largely IFN-γ dependent and synergistic responses are obtained when IL-12 is combined with IL-2, IFN-α or chemotherapeutic compounds (Brunda, 1995). IL-12-mediated inhibition of angiogenesis also has been indicated as an important component of the antitumour activity of this cytokine (Trinchieri, 1997). A role in the inhibition of vessel formation caused by IL-12 has been suggested for IFN-γ and for the antiangiogenic protein IP-10, whose production is also stimulated by IL-12 (Sgadari et al., 1996). These findings imply that IL-12 may promote an antitumour effect via more than one pathway.

A recent study showed that the systemic administration of a low dose of IL-12 was more effective than local administration in curing mice bearing established metastatic tumours, due to differences in action kinetics and intensity (Cavallo et al., 1999).

Systemic treatment of HER-2/neu transgenic mice bearing preneoplastic lesions with IL-12 markedly delayed the onset of mammary gland tumours, which normally develop in these mice, and reduced tumour multiplicity (Lollini et al., 1999). Interestingly, similar inhibition of tumour development was observed in immunocompetent and $CD8^+$ T cell-depleted mice, suggesting that IL-12-induced stimulation of nonspecific immunity can also prevent tumour formation.

The encouraging results in animal models have prompted the use of IL-12 in carcinoma patients. Recently, clinical trials have been performed in order to analyse IL-12-related toxicities and potential methods of their prevention (Golab et al., 1999).

1.3.3 ACTIVE IMMUNOTHERAPY OR VACCINATION

The classical concept of the vaccine derives from the practice of immunising against infectious agents to prevent disease by generating a humoral immunity. In contrast to prophylactic vaccines against infectious agents, the major focus in cancer vaccine development has been on the generation of antigen-specific T-cell responses. Furthermore, in contrast to the limited number of defined antigens for viruses and bacteria, in the case of most tumours there is an unlimited number of potential antigens that can be the target of an immune response. It is also likely that many of these antigens arise during or as a result of the tumourigenesis process.

The most common approach to enhance immunogenicity of an antigen is to mix tumour cells or the antigen preparations with non-specific immune adjuvants. Widely used adjuvants include alum (aluminum salts), bacterial organisms such as BCG (Bacillus Calmette Guèrin) or *Corynebacterium parvum*, components of bacterial cell walls, and Freund's adjuvant. Non-specific adjuvants induce local inflammatory infiltration of APC, T- and B-cells which produce cytokines and chemokines responsible for local microenvironment promoting induction phase of an immune response to the delivered antigens.

Tumour vaccines have not so far been employed to prevent cancer in healthy individuals. They are used mostly in experimental studies in patients with established malignancies as adjuncts to primary treatment, or in clinical trials in patients with advanced disease with high risk of progression into incurable disseminated disease. Immunogenic tumours with known history of spontaneous regression and resistant to other available therapies, such as melanoma or renal cell carcinoma, have been most extensively treated with tumour vaccines.

First generation vaccines made of whole cancer or tumour-cell lysates together with non specific adjuvants produced about 20% of clinical response and are currently tested in prospective clinical trials. Novel second generation vaccines employ tumour cells or antigen presenting cells genetically modified by gene transfer technology (see chapter 1.4). These vaccines actually are mostly tested in toxicity-exploratory phase I/II trials.

1.3.3.1 Autologous or allogeneic whole cell or cell lysate vaccines

Almost all vaccine trials carried out before 1990 involved melanoma patients treated with autologous or allogeneic cell line preparations.

Autologous vaccines consisted of tumour cells isolated from fresh, cryopreserved tissue or the cell line established from autologous tissue. Tumour cells contain unique mutated antigens, which might be directly presented in the context of the host HLA molecules. Autologous vaccines have had many disadvantages: firstly, production of a customised vaccine is time-consuming, and dependent on the quantity of available tumour tissue. Secondly, naturally evolving tumour cells are generally not immunogenic, display defects in expression of HLA and co-stimulatory molecules and may produce immunosuppressive factors which limits their ability to induce specific immune responses.

Allogeneic vaccines consisted of *in vitro* established tumour cell lines. They can be partially characterised by expression of known-shared antigens and if mixed they display a broad range of antigens, thus diminishing the probability of immune escape. Vaccines of cultured established allogeneic cell lines are also much easier to prepare and manufacture.

Allogeneic and autologous cell vaccines have been used in the first large-scale clinical trials of tumour vaccines.

Five clinical responses were observed in a group of 40 evaluable patients after treatment with low-dose of cyclophosphamide, which reduce suppressor cell activity and enhance antibody responses, followed by autologous vaccine with BCG as adjuvant (Berd et al., 1986; Berd et al., 1990).

In another study, three complete and six partial responses were observed in 40 patients vaccinated with three allogeneic melanoma cell lines mixed with BCG (Morton et al., 1993; Morton et al., 1992).

Twenty objective responses in 106 melanoma patients treated with allogeneic cell lysate vaccine mixed were observed. CTL assays and certain HLA phenotypes correlated with clinical responses (Mitchell et al., 1988; Mitchell et al., 1993).

A polyvalent melanoma vaccine prepared from the material shed into culture medium by four melanoma lines bound to alum resulted in 50% overall 5-year survival in 94 treated melanoma patients with stage III disease (Bystryn, 1993; Bystryn, 1995).

1.3.3.2 Protein- and Peptide-based vaccines

The aim of protein- and peptide-based vaccine strategies is to administer high doses of antigen peptide that can be loaded onto empty MHC molecules on APCs *in vivo*. Variable results have been observed: whereas vaccination with some tumour-associated peptides and proteins has induced systemic antitumour immunity (Mandelboim et al., 1995), the administration of other peptides has led to the induction of tolerance rather activation (Toes et al., 1996).

Phase I clinical studies of peptide vaccines have been initiated for the treatment of melanoma. As for whole-cell vaccines, the only toxicities observed have been grade I/II induration and erythema at the vaccine site.

One trial of an HLA-A1-restricted MAGE3 peptide reported three partial remissions in 16 patients with metastatic melanoma at pulmonary and subcutaneous sites (Marchand et al., 1995).

In another study, all three patients with metastatic melanoma demonstrated partial remissions that were associated with DTH reactivity at the vaccine sites after vaccination with three melanoma-associated HLA-A2-restricted antigenic peptides (MART-1/MelanA, tyrosinase and gp100), given simultaneously with GM-CSF (Jager et al., 1996).

A more recent study evaluated an HLA-A2-restricted gp100 peptide analogue, which was modified at one of the MHC anchor residues to improve its binding affinity to HLA-A2, in patients with melanoma (Parkhurst et al., 1996). Immunisation with the anchor-modified peptide in incomplete Freund's adjuvant induced gp100- and HLA-A2-restricted melanoma CTLs in 10 of 11 patients and a clinical response was observed in 42% of patients who received the vaccine along with systemic IL-2.

1.3.3.2 (1) Heat Shock protein as a carrier of antigens

A novel peptide vaccine approach uses heat shock proteins (HSPs). HSPs, or chaperonins, are natural biologic adjuvants found in most cells, which have the capacity to bind a wide array of peptides (Lammert et al., 1997). Their ability to elicit antitumour immune responses has been tested in some studies.

In particular, immunisation with tumour-derived HSP96, an endoplasmic reticulum resident, and HSP70, a cytosolic HSP, revealed the stimulation of a tumour-specific immunity in mice (Tamura et al., 1997). Furthermore, more recently, vaccination with recombinant HSP70 associated to a model peptide antigen was shown to induce antigen-specific CD8+ T cell responses (Suzue et al., 1997). The tumour-specific immunity appeared to be mediated by specific peptides, which are found bound to HSPs

(Blachere et al., 1997). It is postulated that HSPs induce a specific CTL response by introducing antigens into the MHC class I and II pathways as well as by binding to macrophages, thereby inducing the production of proinflammatory cytokines.

1.3.3.3 Carbohydrate antigen vaccines

Carbohydrate antigens aberrantly or overexpressed on tumour cells are further targets for immunotherapy.

Gangliosides GM2, GD2 and GD3, which are cell surface constituents of melanomas and other tumours of neuroectodermal origin, have been used as immunogens in patients with cancer.

For example, a trial comparing cyclophosphamide plus BCG versus cyclophosphamide plus BCG/GM2 ganglioside was carried out in 122 stage III melanoma patients. Vaccine containing GM2 induced IgM responses in 86% patients (Livingston et al., 1989). A pilot study with GM2 conjugated to keyhole limpet hemocyanin as a carrier protein plus an adjuvant produced IgM, IgG1 and IgG3 responses in all six immunised patients (Helling et al., 1995). Expression of the carbohydrate moiety sialyl Tn (STn) is associated with a worse prognosis in colonic (Itzkowitz et al., 1990), gastric (Ma et al., 1993) and breast cancers (Kinney et al., 1997). Circulating antigen has been detected in gastrointestinal and ovarian malignancies and raised levels have been associated with a worse prognosis (Kobayashi et al., 1992). A clinical trial using STn conjugated to keyhole limpet hemocyanin in patients with breast cancer has recently been reported (MacLean et al., 1996). All patients immunised with STn-KLH vaccine generated an antibody response to STn, STn-positive mucin and KLH. A therapeutic effect was observed in the group of patients pre-treated with cyclophosphamide.

1.3.3.4 Anti-idiotypic antibodies vaccines

Apart from direct therapy of cancer with tumour-specific antibodies, a vaccination strategy using antibodies has been proposed. This approach is based on the theory that anti-idiotypic antibodies that bear the internal image of tumour antigens may serve as immunogens. Anti-idiotypic antibodies recognise idiotopes located on variable region of the antibodies, thus they may share conformational or primary structure with the corresponding antigen in order to fit the same combining site, just as two keys that fit the same keyhole display similar shapes.

Anti-idiotypic antibodies that bear internal images of tumour antigens have been developed for several types of tumours. Clinical trials have been carried out in patients with advanced melanoma using anti-idiotypic antibodies that mimicked high-molecular weight-melanoma associated antigens (HMW-MAA), GD3 and GM3. In particular, in a study carried out in 52 patients treated with anti-idiotypic mAb (HMW-MAA) with BCG, a significant increase in survival time was observed in the 18 patients who developed anti-HMW-MAA antibodies (Mittelman et al., 1994; Perera, 1990).

1.3.3.5 Dendritic cell vaccines

Based on the emerging concept of the central role of APCs in the initiation of immune responses, dendritic cell (DC)-based vaccines are under active investigation.

Many factors appear to be responsible for the unique potency of DCs in activating T cells. Firstly, they express 50-fold higher levels of MHC molecules than macrophages, providing more peptide/MHC ligand for T cell receptor engagement. Furthermore, they express extremely high levels of important adhesion molecules, costimulatory molecules and DC-specific genes, such as one encoding a T cell specific chemokine (Adema et al., 1997b), critical for T cell activation.

Either directly isolated or GM-CSF-induced DCs have been used as antigen carriers for tumour vaccination. The form of antigen loaded onto DCs was represented by minimal MHC class I-restricted peptides (Mayordomo et al., 1995), or by the protein antigen (Hsu et al., 1996; Paglia et al., 1996). Moreover, in other cases, the antigen has even been presented by fusion of DCs with whole tumour cells (Gong et al., 1997).

Clinical testing using DC vaccines have been performed. In one study, vaccination of B-cell-lymphoma patients with DCs loaded with idiotypic antibody generated idiotype-specific immunity with some clinical response (Hsu et al., 1996). A recent vaccine trial in melanoma patients using autologous DCs pulsed with either tumour lysate or MHC class I-restricted melanoma peptides, together with keyhole limpet hemocyanin as a helper antigen, reported induction of delayed type hyper sensitivity and some clinical response (Nestle et al., 1998).

1.4 CANCER GENE THERAPY

A promising approach in cancer therapy involves gene therapy, which consists of the introduction of genetic material into a patient' tissues with the intent to achieve therapeutic benefit. Different strategies have been developed for the treatment of cancer; in particular, current efforts focus on:

- immuno-gene therapy, which involves the introduction of genes in order to modulate the patient's immune response capacity;
- chemo-gene therapy, which is the introduction of genes that confer susceptibility to chemotherapeutics;
- correction of genetic defects in tumour cells, such as the introduction of tumour suppressor genes;
- manipulation of tumour growth and invasiveness by introduction of genes that inhibit the angiogenesis process.

In the last years, a number of these approach have entered initial clinical testing.

In the context of this thesis, particular attention will be focused on gene transfer as an immunotherapy approach, followed by a less extensive treatment of the other approaches. Of crucial importance for all these areas still is the development of efficient gene targeting and delivery system.

1.4.1 GENE TRANSFER STRATEGIES

Cancer gene therapy strategies can be classified broadly according to the physical location where genetic modification of target cells takes place (Miller, 1992a).

In an *ex-vivo* approach, target cells are removed from the patient, established in culture, transduced with an appropriate vector, and returned to the patient.

This strategy relies on the ability to harvest the relevant target cells efficiently, to manipulate them in culture without inducing significant alterations of their biologic properties, and to transplant them in such a fashion that they perform their intended function *in vivo*.

An alternate approach, designated *in-vivo* approach, requires the transduction of sufficient numbers of relevant target cells *in situ*. This scheme offers a number of potential advantages over the *ex-vivo* approach, including the need for fewer manipulations of target cell, the simpler administration of therapy and cost reductions.

1.4.2 METHODS OF GENE TRANSFER

An ideal delivery system should be characterised by the following features: protection and delivery of DNA into cells efficiently, preferable to a specific cell type; be non-toxic and nonimmunogenic; be easily produced in large quantities. No existing system meets all the requirements and no single vector has emerged as being optimal for all gene therapy applications.

Current systems differ in their suitability for *ex-vivo* versus *in-vivo* strategies, their capability for persistent gene expression and possible toxicities.

Gene delivery systems currently employed may be divided into two major groups: non viral and viral methods.

1.4.2.1 Non- viral methods of gene transfer

1.4.2.1 (1) Naked DNA

In non-viral systems the therapeutic gene is placed into a DNA plasmid and delivered to the cell of interest. Plasmid DNA transfects both dividing and non-dividing cells; it does not integrate with the genome of the target cell and results in transient gene expression. No limits in the size of therapeutic DNA, low immunogenicity and safety are the most important advantages of this system (Wolff et al., 1990).

Naked DNA encoding cytokines most commonly is injected directly into the tumour or, in the case of genes encoding tumour antigens, into muscles, in which the translated proteins appear to be processed efficiently by host professional antigen-presenting cells and then presented to T-lymphocytes (Doe et al., 1996; Ulmer et al., 1993).

Measures to improve naked DNA transfer efficiency include complexing the DNA with various compounds.

1.4.2.1 (2) Liposome complexes

DNA can be admixed with various formulations of cationic liposomes to form a lipid-DNA complex with a net positive charge, thus favouring fusion with the cell surface membrane (Gao et al., 1995).

DNA-liposome complexes have been used successfully for *in vivo* gene transfer to tumours in animal models and also in clinical trials (Nabel et al., 1993; Plautz et al., 1993). Liposomes may also be used for systemic delivery of therapeutic genes to disseminated lesions. An intravenous injection of DNA-liposomes resulted in expression of a reporter gene in various organs which was detectable for several months (Zhu et al., 1993; Liu et al., 1995; Thierry et al., 1995) and intravenous administration

of liposome-complexed DNA encoding GM-CSF achieved therapeutic levels of the cytokine in the circulation (Liu et al., 1995).

1.4.2.1 (3) Conjugate vectors

Complexes that consist of DNA conjugated with a cell-specific carrier molecule have the advantage of direct gene transfer to defined cell populations by receptor-mediate endocytosis. A cationic molecule such as polylysine is used to bind DNA by ionic interaction. The polylysine molecule is also linked to a ligand, for example transferrin, able to bind to a cell surface receptor, resulting in endocytosis of the DNA-polylysin-ligand complex.

In order to enhance gene transfer efficiency, DNA endocytosed in this way may be protected from degradation in lysosomes by linking the complexes to adenovirus particles. Capsid proteins of adenoviruses are able to mediate disruption of endosomes, thus allowing the DNA to escape degradation (Curiel et al., 1991; Wagner et al., 1992). Although strategies employing DNA-polylysine-ligand complexes have proved to be extremely efficient *in vitro*, probably the large size of these particles may impede the widespread dispersion of DNA complexes *in vivo*, making this approach less effective for systemic gene delivery (Ledley, 1995).

1.4.2.1 (4) Particle bombardment

DNA coated on microparticles may be projected into cells using a "gene gun". DNA is first coated onto the surface of 1-3 micron diameter gold or tungsten beads. These particles are accelerated by an electric discharge device, or gas pulse, and fired at the tissue. Such an approach has been used to transfer various cytokine genes to tumour cells *in vivo*, resulting in inhibition of tumour growth and even complete eradication of tumours in some animals (Sun et al., 1995; Rakhmilevich et al., 1996). Furthermore,

delivery of DNA encoding tumour antigens by gene gun has been shown to successfully elicit protection against tumour (Irvine et al., 1996). Although these positive results prove this strategy useful for superficial lesions, the depth of penetration is limited. Thus, it is unlikely that such an approach would be suitable for widespread systemic delivery of therapeutic genes.

1.4.2.2 Viral vectors

The natural ability of viruses to infect eukaryotic cells and to introduce their genomes, as part of their life cycle into the host cell's genome, is utilised to transfer genetic material into target cells.

Limitations of this system are represented by the size of therapeutic gene (usually, 5-10 kb depending on the vector used), time consuming and expensive preparations. Different viral systems have received attention for gene transfer studies.

1.4.2.2 (1) Retroviruses

Of the viral-based vectors, retroviruses have been the most widely used in experimental studies. Retroviral vectors are also being employed in some clinical trials. They are extraordinarily efficient gene delivery vehicles; in the nucleus the retroviral nucleic acid becomes integrated into chromosomal DNA, ensuring its long-term persistence and stable transmission to all future progeny of the transduced cell.

Retroviruses are divided into three subfamilies, based primarily on pathogenicity: oncoviruses, lentiviruses and spumaviruses. The oncoviruses are further subclassified into 5 groups, A to D, largely on the basis of their morphology and method of release from cells. The group which has been used most extensively to derive retroviral vectors are the murine C-type oncoviruses.

Murine C-type viruses have been classified into the following groups, based on the host range of the virus, interference patterns and serotype of the envelope antigen (Weiss et al., 1995):

- ecotropic, which infect mouse and rat cells,
- amphotropic, which infect mouse and other cells
- xenotropic, which do not infect mouse cells but are able to infect other cells
- dual-tropic/polytropic, which infects mouse and mink cells.

i) Structure

A mature retrovirus comprises an inner core enclosed in a phospholipid envelope. The core consists of an icosahedral protein shell, the capsid, separated from the envelope by matrix protein, which houses the retroviral genome. The genome consists in two copies of a positive sense RNA, carrying a cap at its 5'end and a poly(A) structures at its 3'. The core also contains virally-encoded protease, reverse transcriptase and integrase enzymes. The envelope is a roughly spherical phospholipid bilayer derived from the plasma membrane of the virus-producing cell and is covered with closely packed oligomeric membrane spike glycoproteins, which appear as surface projections on electron microscopy (Fig. 1.11).

ii) Life cycle

The retroviral life cycle has been studied extensively (Varmus, 1988) (Fig. 1.12).

The viral envelope glycoprotein (env) attaches the virus to its cell surface receptor and catalyses a membrane fusion event which releases the viral core into the cytoplasm of the target cell.

Several of the cell surface receptors which are used by retroviruses have been identified, some of which are transporter molecules which serve normal cellular functions.

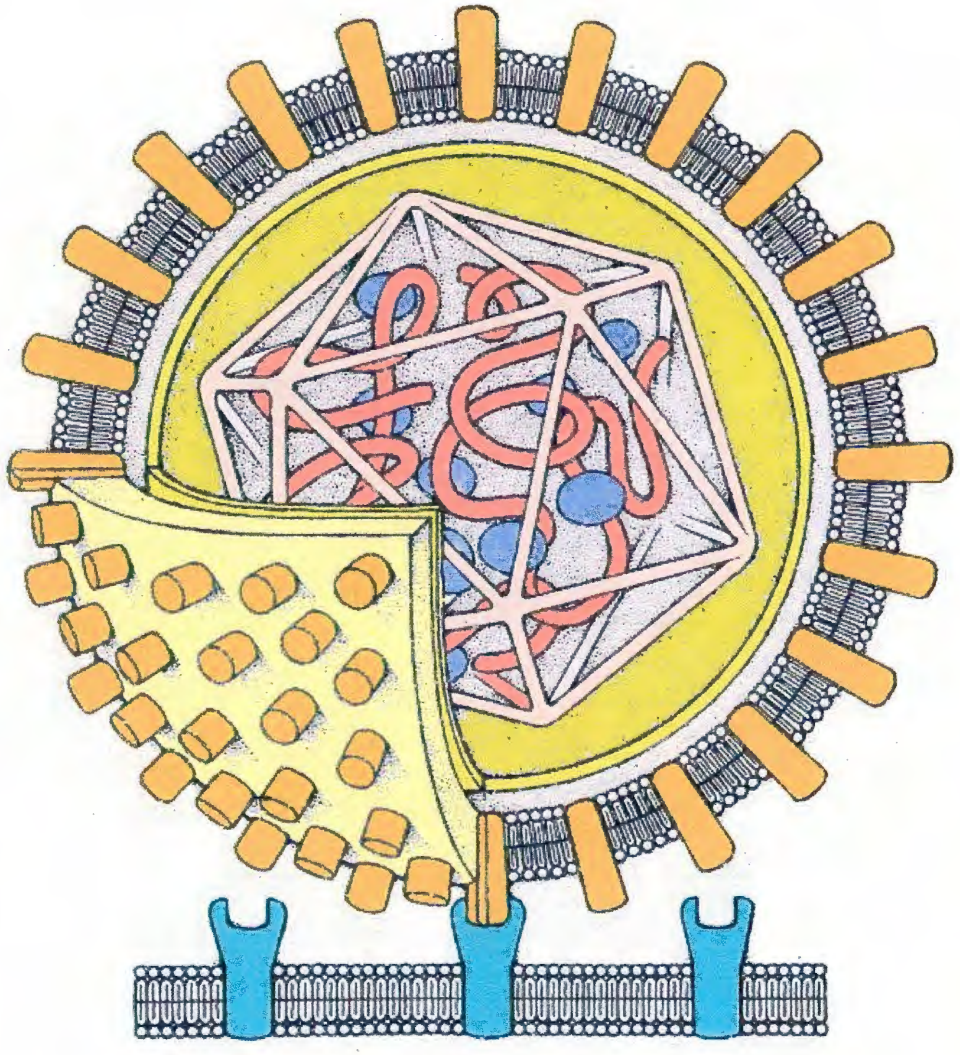


Fig. 1.11: Schematic view of a retroviral particle

Two identical single strands of viral RNA and viral enzymes (reverse transcriptase, integrase and protease) are drawn within an icosahedral viral core and the core is surrounded by an envelope that is derived from host membranes enriched with viral glycoprotein. Interaction of envelope glycoprotein with a host-encoded cell surface receptor is shown at the bottom (modified from Varmus, 1988).

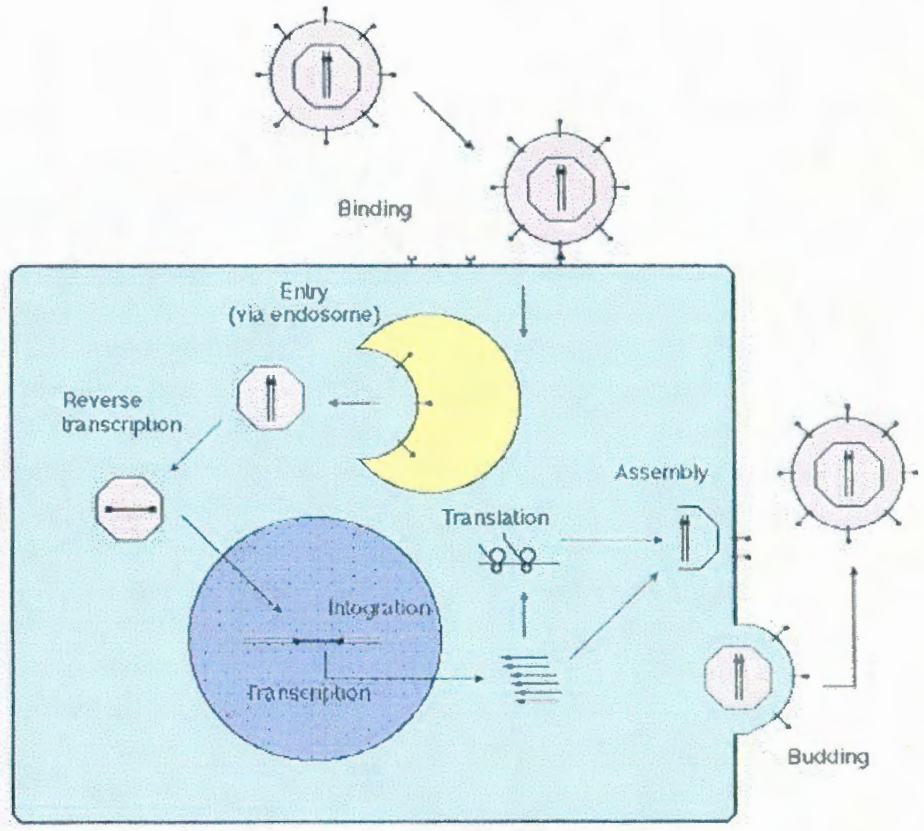


Fig. 1.12: The retroviral life cycle

A virus particle entering a cell, uncoating to form a nucleoprotein complex in which viral RNA is transcribed into DNA by reverse transcriptase. After migration to the nucleus, the complex mediates integration into a host cell chromosome. Synthesis of viral RNA and proteins leads to assembly of particles that exit the cell by budding through the plasma membrane (modified from Vile et al., 1995).

For example, the Murine Leukaemia Viruses (MLVs) ecotropic receptor has been identified as a cationic amino acid protein (Albritton et al., 1993) and the MLV amphotropic receptor is an inorganic phosphate transporter, Ram-1 (Miller et al., 1994). Cells infected with one virus or expressing the viral envelope glycoprotein of that virus impede superinfection with another virus bearing a similar envelope by competitive blocking of receptors. This phenomen is known as receptor interference (Sommerfelt et al., 1990).

The RNA genome is reverse transcribed by a complex mechanism to double-stranded (proviral) DNA by reverse transcriptase, which is contained in the core of the virus (Fig. 1.13). A cellular tRNA is packaged with in the virus; this tRNA acts as a primer for synthesis of the minus strand DNA by annealing to the primer binding site (pbs). Elongation of the minus strand proceeds through the U5 and R region. The RNA template is then removed by the RNAse activity of reverse transcriptase (RNAse H), thus allowing the exposed DNA to bind to the complementary R region at the 3'end of the genome. Synthesis of the minus strand DNA then continues. Synthesis of the plus strand DNA requires RNAse H to cleave the RNA at a specific site (ppt), thus allowing the nicked RNA to act as a primer. The plus strand is synthesised through the U3, R and U5 regions, using the minus strand DNA as a template, and continues into a portion of the primer tRNA. The plus strand DNA then transfers to the pbs site at the 3' end of the minus strand DNA and synthesis of both strands is then completed, forming a double-stranded copy with long terminal repeats (LTR) consisting of U3-R-U5 at both ends.

The double-stranded DNA is then transported to the cell nucleus and integrates, after capsid disassembly, into a random chromosomal site. This process is mediated by the viral enzyme integrase.

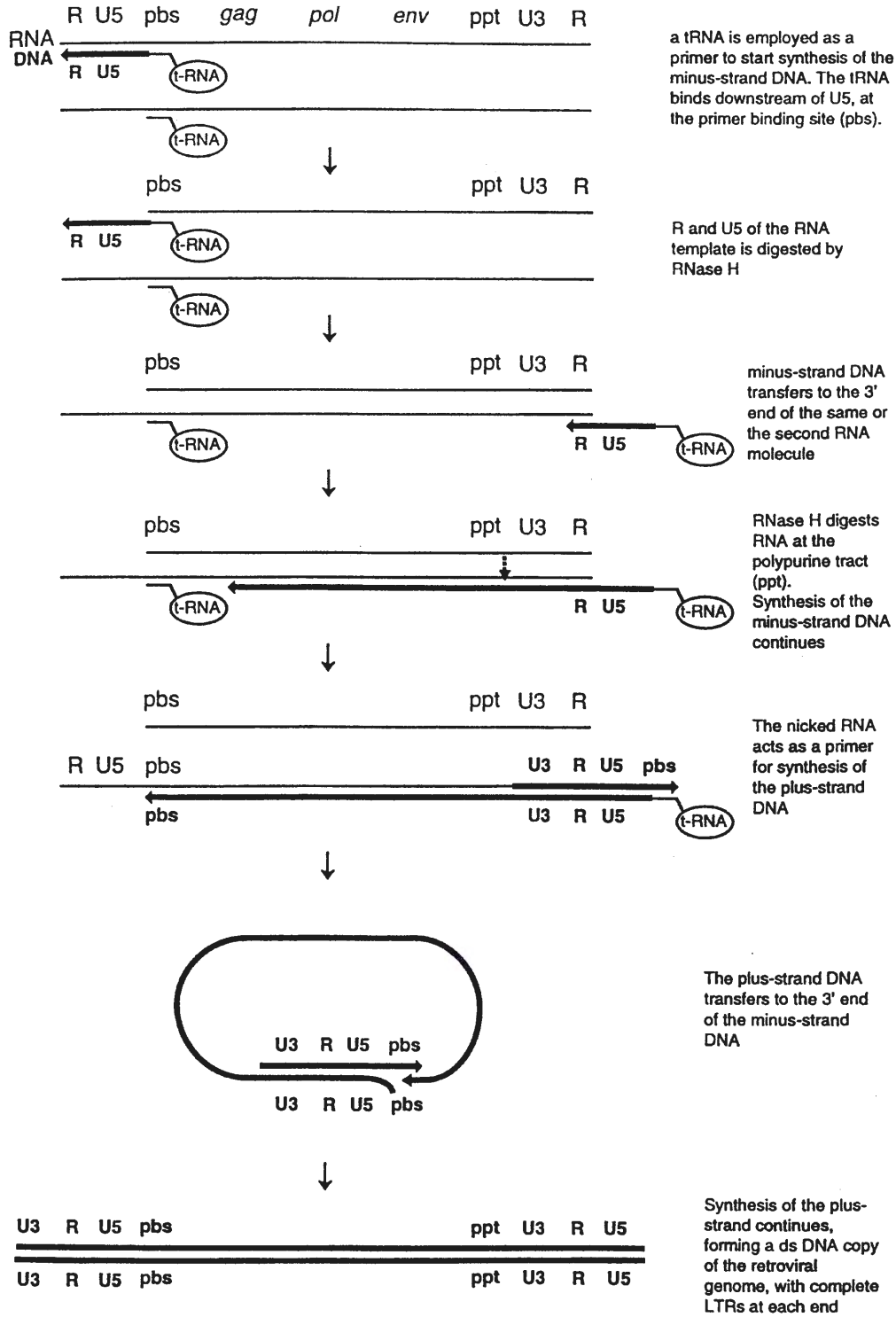


Fig. 1.13: Generation of a DNA copy of retroviral genome

The RNA genome is reverse transcribed by a complex mechanism to double-stranded (proviral) DNA by reverse transcriptase.

Capsid of C-type retroviruses cannot traverse the intact nuclear membrane and cell mitosis therefore is required for successful progression of the viral life cycle. Retroviral integration is non-homologous, with some degree of sequence specificity (Shih et al., 1988) and a preference for transcriptionally active host integration sites (Mooslehner et al., 1990).

After integration of the double stranded DNA provirus, regulatory promoter and enhancer elements in the U3 region of the proviral long terminal repeat (LTR) drive transcription of the viral genome (Fig. 1.14). The full lenght transcript encodes the gag and gag-pol polyproteins, while a smaller, spliced transcript encodes the env proteins. Oligomerisation of the gag and gag-pol polyproteins to form budding viral core particles is central to the assembly and release of fully infectious viral progeny. Full-length (unspliced) viral mRNA binds, through its packaging signal sequence (Psi), to the gag polyprotein during particle assembly and env glycoproteins, present at the plasma membrane, are incorporated into viral progeny, as they bud from the cell surface. Newly budded virions are not infectious until the viral protease cleaves the gag and gag-pol polyproteins into their component parts.

iii) Retroviral vectors

Various retroviruses have been used as vectors (Miller, 1992b), but those based on Murine Leukaemia Viruses (MLVs) are the most advanced and have been most widely used to date.

Most human gene therapy applications require replication defective retroviral vectors capable of delivering therapeutic genes to individual target cells, without further replicative spread.

The production of replicative-defective retroviral vectors involves two main components: a vector and a packaging cell line (Fig. 1.14).

The vector is constructed from a retroviral genome from which the protein coding sequences (gag, pol and env) are removed and replaced by heterologous coding sequences. The viral functions required for replication are provided *in trans* by the packaging cell line.

Some elements need to be retained *in cis* by the vector, to enable it to be packaged, reverse transcribed and integrated into the chromosome of the target cell. These elements include:

- the packaging signal sequence, which ensures the encapsidation of the vector RNA into virions (Psi). More recent vectors retain an extended Psi sequence, which incorporates the start of the gag gene (Psi^+), with the AUG start codon of the viral gene mutated. The presence of this region has been demonstrated to increase the efficiency of packaging, resulting in an increase in viral titre (Bender et al., 1987).
- elements necessary to direct the process of reverse transcription, including the primer binding site (PBS), which binds the tRNA primer of reverse transcription; the terminal repeat (R) sequences, that guide the "jumping" of the reverse transcriptase between RNA strands during DNA synthesis and a purine-rich sequence in the 5' region of the 3'LTR that serves as the priming site for synthesis of the second (plus) DNA strand.
- Specific sequences near the ends of the LTRs, that are necessary for the integration of the vector DNA into the host cell chromosome in the ordered and reproducible manner characteristic of retroviruses.

Up to 8 kilobases of foreign gene sequence can be packaged in a retroviral vector, since a too large vector-derived RNA transcript cannot be packaged efficiently into retroviral particles. Adhering to this size constraint, it is possible to insert 1, 2, or even 3 genes into the vector genome in forward or reverse orientation, controlled by viral or non-viral promoter and enhancer sequences.

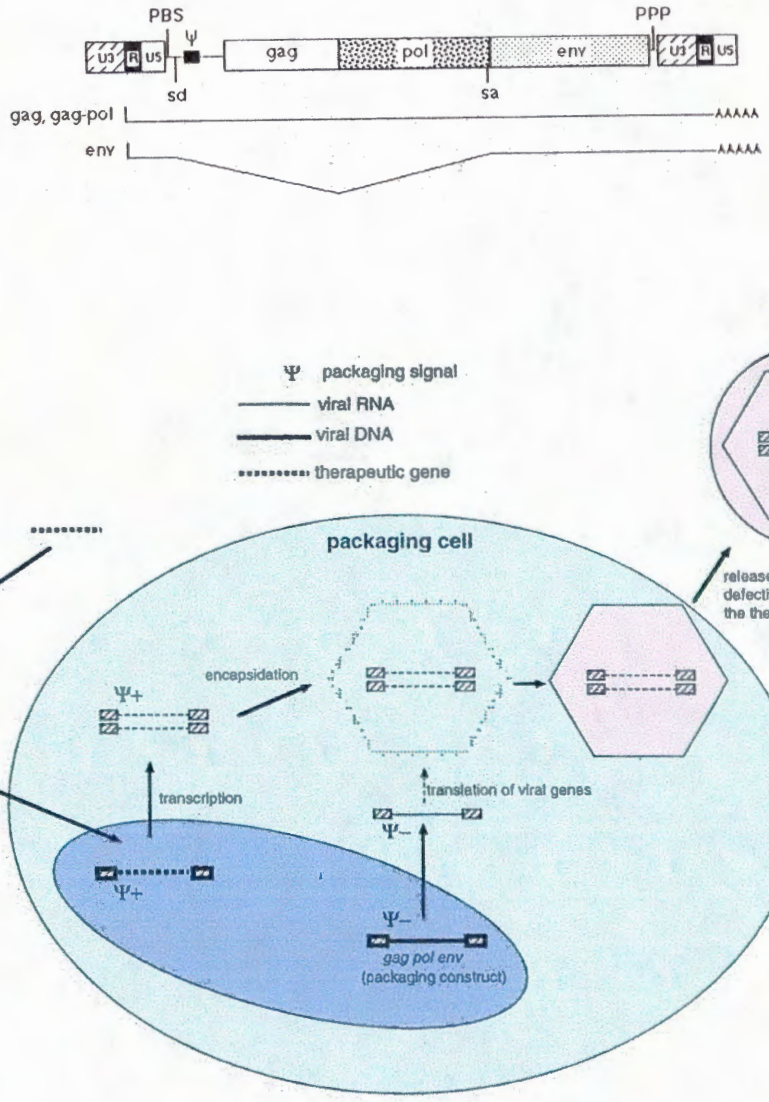


Fig. 1.14: Generation of retroviral vectors

Structure, transcription and splicing of Moloney MLV provirus: PBS=tRNA primer binding site; Psi=packaging signal sequence; PPP=polypurine tract or plus strand primer binding site; sd=splice donor; sa=splice acceptor

A retroviral packaging cell line: the cell line constitutively produces empty retroviral particles lacking an RNA genome; RNA molecules carrying the packaging signal sequence (derived from the vector construct) are efficiently packaged (from Vile et al., 1995).

iv) Retroviral packaging cell line

The purpose of the retroviral packaging cell line is to provide the viral helper functions, the gag, pol and env proteins, which have been deleted from the vector genome. These helper functions are stably expressed in the packaging cells from one or more plasmids. When a vector genome is transfected into such packaging cells, the viral gag proteins recognise and package the vector RNA genome, which is provided by the packaging signal sequence, into viral particles. These viral particles are then released into the culture supernatant.

It is essential for employment in clinical trials that retroviral vector stocks are not contamined with replication competent retrovirus. The helper and vector sequences introduced into a packaging cell carry all the viral genes and regulatory sequences required to reconstitute a wild type viral genome. The greater the sequence homology between helper and vector genomes, the higher is the chance that they will align and undergo homologous recombination.

The earliest first generation packaging cell lines consist of cells containing a retroviral genome deleted only in the Psi region (pMOV-Psi^-) (Mann et al., 1983). These cells provide functions needed for viral replication and produce empty viral particles which contain reverse transcriptase, but since the transcript produced from the helper construct are Psi^-, they are packaged at very low frequencies. However, the Psi^-RNA transcripts, derived from packaging cell line, were shown to be packaged with Psi^+ vector transcripts at low frequency (0.1%) into budding retroviruses and replication competent retrovirus most frequently arose by recombination of the co-packaged transcripts during reverse transcription. Examples of first generation packaging lines are ψ2 (Mann et al., 1983), ψ-AM (Cone et al., 1984) and PA12 (Miller et al., 1985).

The best way to reduce the risk of replication competent retrovirus is to provide the helper functions on more extensively deleted helper plasmids having minimal sequence homology with the vector genome, or to split them onto multiple helper plasmids.
Second and third generation packaging cell lines have been developed according to these principles and are considerably safer, due to increased number of helper-vector recombination events required to generate a wild type viral genome.
PA317 (Miller et al., 1986), a second generation packaging cell line which has been used to produce retroviral vectors for human clinical trials (Rosenberg et al., 1990), was generated using the plasmid pPAM3, which lacks not only the packaging sequence but also the 3'LTR and polypurine tract, which have been replaced by an SV40 polyadenylation signal sequence. Two recombinations between helper and vector sequences are therefore necessary to regenerate replication competent retrovirus and this double recombination has proven to be a very low frequency event.

Third generation packaging cells have further reduced the risk of replication competent retrovirus, by separating the gag-pol transcription unit from the env transcription unit on different plasmids and by using packaging constructs which have minimal areas of homology with the vector genome (Miller, 1990). This arrangement excludes the possibility that two co-packaged constructs can recombine to generate replication competent retrovirus, since the entire viral genome is now split three ways onto the vector construct and the two helper constructs. Examples of these cell lines, some of which are used in this thesis, are GP+E86 (Markowitz et al., 1988a) and ψCRE (Danos et al., 1988), which produce viruses with ecotropic host range, and GP+envAM12 (Markowitz et al., 1988b) and ψCRIP (Danos et al., 1988), which release vectors with amphotropic host range. However, it has been shown that even third generation packaging cell lines may package and transfer endogenous murine viral genomes (Scadden et al., 1990).

More recently, different approaches have been used to improve third generation packaging cell lines.

In order to reduce regions of homology, packaging lines have been constructed which minimise their content of MLV-derived sequences in the helper constructs. The Propak-A (Rigg et al., 1996) and ampli-GPE (Takahara et al., 1992) lines employ heterologous promoters to drive gag-pol and env, as well as using heterologous sequences for the polyadenylation site. In the ampli-GPE line, in order to maximise viral titres, high expression levels of the helper functions are maintained by placing gag-pol and env in separate bovine papilloma virus-based constructs which can be amplified 20 to 50 fold in an episomal state.

Packaging lines which produce viral particles bearing alternative envelopes, such as that derived from gibbon ape leukaemia virus (GALV) have been produced (Miller et al., 1991). GALV pseudotyped viruses were found to be more efficient in infecting human lymphocytes compared with amphotropic viruses. This greater efficiency reflects the higher expression of Glvr-1 relative to Ram-1 in this cell type (Lam et al., 1996; Porter et al., 1996). Moreover, GALV-based packaging lines produce vectors with an extended species host range, including hamster and bovine cells, which are not well infected by amphotropic viruses (Miller et al., 1991).

v) Efficiency of gene delivery and expression

For maximum efficiency of gene delivery, a retroviral vector should be available at high titre and should have unimpeded access to its target cell population.

Under optimal conditions, the most widely used retroviral producer cells can generate 10^6-10^7 infectious vector particles/ml of tissue culture supernatant. The manipulation of vectors with extra promoters and a large transgene has the disadvantage that it reduces viral titres.

A disadvantage of retroviral vectors based on murine leukaemia viruses is their poor ability in introducing genes to non-dividing cells (Miller, et al., 1990). This characteristic may be useful in cancer therapy, since the vectors will then be targeted preferentially to the tumour cells, which proliferate more actively than neighbouring normal host cells. Nevertheless, even in fast-growing tumours, only a small proportion of cells is actively dividing at any one time, leaving many cells uninfected.

Cells infected with retroviral vectors and maintained *in vitro* usually show constant levels of gene expression for long periods of time. However, when cells are returned *in vivo* they often lose expression of the foreign gene over a period of a few weeks. This *in vivo* instability may be due to methylation (Richards et al., 1993; Palmer et al., 1991) or deletion (Russell et al., 1991) of the proviral DNA. A strategy to obviate this problem has been the use of a cellular promoter to drive expression of the exogenous gene, since it might be expected that such promoters would be less likely to be down-regulated compared with viral promoters (Rettinger et al., 1994). While, in some cases, encouraging results using cellular promoters have been observed, in others, problems due to interference effects between promoters (Salmons et al., 1993), as well as a methylation inducing a shutdown of promoters, have been obtained (Richards et al., 1993).

A major difficulty with the use of retroviral vectors for *in vivo* gene therapy in humans is that MLV-based vectors are inactivated rapidly by human complement (Welsh et al., 1975). Sera from Old World Primates, including humans, contain an anti-a-galactosyl antibody, the target epitope of which is formed by Gal(α1-3)Gal terminal carbohydrates present on the surface of most mammalian cells. This epitope is not present on human cells, because of the lack of a functional (α1-3) galactosyltransferase gene in humans (Takeuchi et al., 1996). Therefore, retroviruses produced from non-primate cells acquire

this epitope on their envelope, are recognised by the antibody and become inactivated. In order to produce viral vectors resistant to human serum, packaging lines such as FLYA13, FLYRD18 (Cosset et al., 1995) and Propak-A (Rigg et al., 1996) were derived from human cell lines.

vi) Targeting of retroviral gene transfer

In the interests of safety and efficiency, it is desiderable that retroviral gene delivery should be accurate: the therapeutic genes should be delivered exclusively to the target cells and should integrate into a selected chromosomal location. Different strategies have been evaluated. One approach is represented by the incorporation of tissue-specific or tumour-specific elements in the vector to drive the expression of transgene (Vile, 1994). The tyrosinase gene promoter has been used to target expression to disseminated melanoma cells (Vile et al., 1993; Vile et al., 1993), while the prostate-specific antigen gene promoter has been used to target expression to prostate carcinoma cells (Pang et al., 1995).

Furthermore, promoters of genes overexpressed in target tumour cells also have been used. For example, the a-fetoprotein gene promoter has been used to target expression to hepatoma cells (Huber et al., 1991) and the oncogene c-ERBb2 promoter has been used to restrict expression to tumour cells overexpressing this oncogene (Harris et al., 1994).

As a different approach, attempts to target binding of retroviral vectors to specific cell type have been made by modifying the viral envelope (Salmons et al., 1993). For example, the ligand for HER-2 has been incorporated into the viral envelope glycoproteins to target retroviral particles to breast carcinoma cells, which overexpress HER-2 (Han et al., 1995).

1.4.2.2 (2) Adenovirus

The most obvious advantages of adenoviral mediated gene transfer approaches are their safety and their ability to infect almost all cell types, dividing as well as quiescent cells, *in vitro* and *in vivo*, with high efficiency. Furthermore, adenoviral vectors can accept large foreign DNA inserts, up to 7-8 kb, and for *in vivo* gene transfer high titres, of up to 10^{11}/ml, may be achieved. Adenovirus rarely integrates into the host genome and therefore has little chance to activate cellular oncogenes. The adenoviral vector, once inside the nucleus, remains as a nonreplicating extrachromosomal entity resulting in a transient expression of the transgene.

Three loosely defined sets of proteins exist in the mature adenovirus: proteins that form the outer coat of the capsid, scaffolding proteins that hold the capsid together and DNA binding protein. Protruding from the capsid is the fibre protein, which mediates the initial attachment of the virus to a target cell.

Adenoviruses contain double stranded DNA as genetic material, functionally divided into 2 major non contiguous overlapping regions, early and late, transcribed before or after viral DNA synthesis, respectively. There are 6 distinct early regions, E1A, E1B, E2A, E2B, E3 and E4, each with individual promoters, and one late region, which is under the control of the major late promoter with 5 well characterised coding units, L1 to L5 (Horwitz, 1990).

Replication-deficient adenoviral vectors are constructed by deletion in the E1A region, which encodes for a transactivator that upregulate transcription from the other early regions. The E1A-deleted viruses are replication-defective and the viruses need to be propagated in a cell line which is able to provide the E1A functions in trans, such as the 293 human embryonic kidney line.

Although designed to be replication-deficient, there is the potential for replication *in vivo*, as a consequence of trans-complementation by E1A-like factors due to pre-existing or acquired adenovirus sequences, or by other viruses and/or host cellular transcription factors. For example, low level replication of recombinant adenovirus has been observed in Hela cells, at high multiplicity of infection (Jones et al., 1979).

In addition to E1, other sequences may also be deleted. For example, E1- and E3-deleted vectors, which can accomodate a larger exogenous gene, have been obtained (Bett et al., 1994).

Despite the fact that expression for more than 1 year has also been observed, duration of expression normally is short (about 8 weeks) in adult animals. Therefore, repeated administration may be necessary.

A number of hypotheses have been proposed to explain this short-duration expression. First of all is that the high immunogenicity of the viral capsid proteins results in the elimination of infected cells by the immune system. A disadvantage of this response is that neutralising antibodies make re-administration of the vector less effective (Yang et al., 1994a; Yang et al., 1996b). Secondly, it is also possible that a slow accumulation of toxic viral-encoded-proteins may kill the cell and/or that the duration of expression may be reduced by intracellular methylation of the viral promoter (Doerfler, 1993).

Strategies to improve adenoviral vectors have been applied. An approach is represented by the creation of a helper virus system that provides all the gene functions from the deleted region. This approach has the potential to delete from the vector all sequences except for *cis* acting elements, that are necessary to package the recombinant adenovirus, allowing the insertion of increased size of DNA and improving the safety features (Fisher et al., 1996). After propagation in 293 cells, the recombinant vector virions and helper viruses may be separated on the basis of differential sedimentation densities in CsCl gradient.

Another approach is represented by the inactivation of viral genes enclosed in the vector by introduction of temperature-sensitive mutations. For example, a temperature-sensitive mutation of the E2A gene, encoding a DNA binding protein, was incorporated into an E1-deleted vector (Engelhardt et al., 1994). The vector may be propagated *in vitro* at a permissive temperature in 293 cells, but *in vivo* E2A is not functional and, in theory, expression of late viral proteins is diminished. This strategy resulted in a more prolonged expression of the transgene *in vivo*.

1.4.2.2 (3) Adeno-associated virus

Another virus that has received considerable attention for gene transfer studies is the Adeno-associated virus (AAV) (Berns KI, 1966). The appeal of this agent derives from its abilities to infect nonreplicating target cells and to integrate into host genome, allowing it to achieve sustained gene expression. Only minimal viral gene products are retained in current vectors, which affords minimal immunologic reactivity.

The virion has a simple structure, composed of 3 unglycosylated proteins and containing a linear single-stranded DNA molecule. For its replication, AAV require coinfection with a helper virus. Without an helper virus, AAV integrate into the host genome and remains as a provirus.

The exogenous gene is inserted between the two terminal repeats, which are the only cis-acting elements that need to be retained in the vector. Viral vector particles are produced from cells by co-transfection of the plasmid vector and a vector bearing the complementing viral proteins, together with co-infection of a helper adenovirus.

1.4.2.2 (4) Other viruses

Several other viruses have been studied for their utility in gene transfer. These include the herpes simplex virus (HSV), which, given its natural tropism, should be suited for

central nervous system applications (Marconi et al., 1996; Roizman, 1996). HSV enters into a lytic pathway which leads to cell death, or, in nervous system cells, may persist indefinitely in a latent state. Attenuated herpes simplex viruses with oncolytic activity against gliomas have been constructed and characterised for residual neurovirulence (Martuza et al., 1991).

Vaccinia viruses are an important tool for tumour vaccination strategies. Soon after the entry into the host cells, these viruses replicate in the cytoplasm to high numbers and are able to produce high levels of transgene expression (Moss, 1996). Presentation of relevant epitopes of the antigen may be facilitated, by direct targeting of heterologous protein to the compartment in which processing of MHC class I antigens is initiated for presentation to $CD8^+$ CTLs. Indeed, mini-genes encoding peptides of 8 to 10 amino acid residues have been attached to sequences that target the peptides for insertion into the endoplasmic reticulum, where the peptides can bind to MHC class I molecules (Restifo et al., 1995). Another study has recently demonstrated the potency of vaccinia vector carrying the Human Papilloma Virus (HPV) gene E7 fused with the LAMP1 gene, a lysosomal-associated membrane protein, which targets E7 to the MHC class II antigen-processing pathway for presentation to $CD4^+$ T cells (Wu et al., 1995; Lin et al., 1996). A recombinant E6- and E7-expressing vaccinia virus has been tested in eight patients with late-stage cervical cancer. No significant side effects were described and an HPV-specific CTL response was observed in one patient. However, all eight patients mounted an anti-vaccinia antibody response (Borysiewicz et al., 1996), which can be the result of either previous immunisations with vaccinia viruses or exposure to cross-reactive viruses. Moreover, recently clinical studies in cancer patients using recombinant vaccinia viruses encoding tumour antigens (Scholl et al., 2000; Marshall et al., 2000) have confirmed the safety and validity of such an approach in stimulating an anti-tumour response.

1.4.3 IMMUNO-GENE THERAPY APPROACHES

1.4.3.1 Genetic modification of tumour cells

Novel second generation vaccines employ tumour cells genetically modified to improve their immunogenicity.

Different strategies have been applied and evaluated in experimental and clinical approaches.

1.4.3.1 (1) Introduction of MHC genes

It has long been known that increased expression of MHC class I proteins on tumour cells enhances their ability to be lysed by tumour-specific cytotoxic T lymphocytes *in vitro*. Studies in the 1980s demonstrated that enhancing MHC class I expression by gene transfection resulted in decreased tumourigenic capacity and/or metastatic capacity of murine tumour models (Hui et al., 1984b; Wallich et al., 1985).

Despite these positive studies, the relationship between levels of MHC expression and tumourigenicity is quite inconsistent when multiple tumour models are studied. Indeed, in some cases, tumour cells expressing high levels of syngeneic MHC class I molecules, by virtue of gene transfer (Mandelboim et al., 1992), appear to be rejected by syngeneic hosts, resulting in an immunisation against low levels of a subsequent parental tumour challenge. Surprisingly, in other cases, MHC class I expression resulted in a complex phenotype, with paradoxical enhancement of metastasis formation, perhaps related to decreased recognition of the transduced tumour by natural killer cells (Glas et al., 1992; Kawano et al., 1986).

While most epithelial tumours express MHC class II products constitutively, the vast majority are MHC class II negative. The introduction of MHC class II molecules in some systems has also resulted in tumour rejection and systemic immunity (Ostrand-

Rosenberg et al., 1990). It was postulated that the expression of MHC class II molecules on tumour cells allowed for the presentation of class II-restricted tumour-specific antigens to helper T cells, which ultimately could provide enhanced *in vivo* help for activation of cytotoxic T cells. The major problem with this hypothesis is that most epithelioid tumour cells do not express co-stimulatory signals, such as the B7 molecule, and, therefore, are not effective antigen-presenting cells.

However, while the introduction of autologous MHC class I and class II molecules into tumour cells may represent an important experimental model, it is important to recognise that because of the polymorphisms in the outbred population, as well as the number of different loci in an individual that could potentially be critical in the presentation of specific tumour antigens, such a genetic approach in humans would necessarily be highly individualised and therefore impratical for treating large numbers of patients.

1.4.3.1 (2) Introduction of the B7 gene

The B7 family of costimulatory molecules, originally described as an activation antigen on B cells, is expressed constitutively by most APCs and is known to be the ligand for two receptors expressed on T cells. These are CD28 and the CTL-associated antigen-4 (Azuma et al., 1993; Freeman et al., 1993b; Freeman et al., 1993a; Linsley et al., 1991; Chen et al., 1992). CD28 is a critical receptor for generating the costimulatory signals in T-cell activation. Cross-linking of CD28 has been shown to enhance the level of lymphokine production by CD4+ T cells subsequent to antigen recognition. In some *in vitro* systems, CD28 engagement not only enhances T-cell activation but is necessary for driving T cells toward activation rather than anergy (Harding et al., 1992).

With this backgroud, the B7 gene was a logical choice for introduction into tumour cells as an approach to enhanced immunisation capacity.

A number of laboratories have indeed shown that transfection of B7.1 into some tumours results in rejection of that tumour and generates systemic immunity against wild-type tumour challenges (Townsend et al., 1993). In other studies, cotransfection of B7.1 with either MHC class II (Baskar et al., 1993), interleukin IL-4 (Cayeux et al., 1995), or IL-7 (Cayeux et al., 1996) has been shown to induce potent systemic immunity.

1.4.3.1 (3) Introduction of the cytokine genes

Genes that encode cytokines are the most common types of genes that have been introduced into tumour cells. The expression of these genes alters the local immunologic environment of the tumour cell so as to either enhance presentation of tumour-specific antigens by APCs or to enhance the activation of tumour-specific lymphocytes. The cytokine is produced at very high concentrations local to the tumour and systemic concentrations generally are quite low. This paracrine physiology much more closely mimics the natural biology of cytokine action than does the systemic administration of recombinant cytokines.

Many cytokine genes have been introduced into tumour cells with varying effects on both tumourigenicity and immunogenicity. Some of these cytokines, when produced by tumour, induce a local inflammatory response that results in elimination of the injected tumour. This local inflammatory response is most often predominantly dependent on leukocytes other than classical T cells. However, in a subset of the studies, the induction of a systemic immune response mediated by T cells has been demonstrated against challenge with the wild type parental tumour.

Given the number of studies done to date with cytokine-transduced tumour cells, it is not surprising that variable results have been seen when different tumour systems are analysed. Additional variables, such as cell dose, level of cytokine expression and

location of immunisation and challenge site, are crucial parameters affecting vaccine efficacy for cytokine-gene engineered tumour cells.

i) IL-2 gene

One of the most extensively studied approaches to cytokine gene transfer into tumour cells is use of the interleukin-2 gene.

A number of studies were performed by transfection of different murine tumour models. They include a Fisher rat cell line, the J558 plasmacytoma, the CT26 colon carcinoma, the B16 melanoma, the CMS-5 fibrosarcoma, the P815 mastocytoma and the HSNLV sarcoma (Bubenik et al., 1988; Bubenik et al., 1990; Fearon et al., 1990; Gansbacher et al., 1990a; Gansbacher et al., 1990b; Ley et al., 1990; Ley et al., 1991; Russel et al., 1991).

Several of these studies showed a marked regression of IL-2 secreting tumours and establishment of a T cell-mediated protection against a challenge with the parental tumour. The inhibitory effect appeared to be dose-dependent, because the degree of suppression of growth correlated directly with the amount of IL-2 produced by the tumour cells and the maximum cure rate of mice was observed to be achieve with intermediate doses of IL-2 (Schmidt et al., 1995; Sobol et al., 1995). *In situ* IL-2 release led to a dense infiltration of the tumour, involving mainly CD4+ and CD8+ T cells and natural killer (NK) cells.

On the other hand, some studies demonstrated that IL-2 secretion could bypass the requirement for T helper function in the generation of tumour-specific cytotoxic T lymphocytes (CTL). Indeed, *in vivo* depletion experiments showed that CD4+ T cells were not necessarily required for rejection of the tumour, while CD8+ T cells appeared to be essential for complete tumour rejection and for long-term protection against the parental tumour (Fearon et al., 1990; Cavallo et al., 1992; Hock et al., 1993b).

IL-2 was reported also to protect against tumour growth when secreted by allogeneic cells, with respect to the tumour and the host animal (Roth et al., 1992). In addition, Fakhrai et al. (Fakhrai et al., 1995) demonstrated that tumour cells admixed with IL-2 transfected bystander cells (fibroblasts) are able to elicit a protective anti-tumour immune response in naïve animals and therapeutic responses in tumour-bearing hosts.

Transduction of the IL-2 gene into human primary melanoma cell cultures (Patel et al., 1994), different human tumours cell lines (Arienti et al., 1994; Cignetti et al., 1994) and human fibroblasts (Veelken et al., 1996) has also been reported by several groups, using retroviral vectors or non-viral-based gene delivery methods. The transduction of IL-2 gene in these human cell lines was demonstrated to increase their capacity to the *in vitro* activate tumour-specific and unspecific lymphocytes lines (Arienti et al., 1994).

ii) IL-4 gene

A number of studies performed in murine tumour models, such as plasmacytoma, adenocarcinoma, melanoma, renal cell carcinoma and sarcoma revealed regression of tumours after transfer of IL-4 transduced tumour cells (Tepper et al., 1989; Blankenstein et al., 1990; Golumbek et al., 1991; Li et al., 1990). The anti-tumour activity of IL-4 was dose-dependent and could be specifically blocked by an anti-IL-4-antibody (Tepper et al., 1992).

The tumour infiltrate showed a massive presence of macrophages and eosinophils, while a small number of T lymphocytes, mostly CD8, infiltrate IL-4-transduced tumour sites only a few days after the initial injection (Golumbek et al., 1991). IL-4 is known as a direct inducer of tumouricidal activity by macrophages (Crawford et al., 1987), while the striking eosinophil infiltrate has been attributed to the induction of VCAM expression on local vascular endothelial cells by IL-4 (Thornhill et al., 1991). VCAM

appears to be the most important ligand for the VLA-4 receptor, expressed at high levels on circulating eosinophils (Schleimer et al., 1992). *In vivo* antibody-mediated depletion of eosinophils resulted in continuous growth of the IL-4 transduced tumour demonstrating the crucial role of eosinophils in tumour elimination (Tepper et al., 1992).

Human tumour-derived stromal cells and fibroblasts have been successfully transfected with the IL-4 gene for clinical vaccination protocols. *In vitro* studies demonstrated that IL-4 transduced stromal cells are able to stimulate proliferation of a patient's PBL (Hunt et al., 1993; Lotze et al., 1994).

iii) IL-12 gene

IL-12, primarily produced by activated macrophages, can promote cytolytic activity, stimulate the production of IFNγ and cause promotion of Th1-type responses. Its effects suggest an important potential in cancer therapy.

Transduction of IL-12 in the CT-26 murine colon carcinoma tumour cells led to delayed tumour onset and an increased survival time (Martinotti et al., 1995). *In vivo* depletion of NK cells resulted in a reduced IL-12 effect on latency or survival, while depletion of CD8+ or CD4+ T cells revealed an unchanged effect on tumour regression. Moreover, while the tumour infiltrate of normal mice showed a minimal T cell contribution, tumour infiltrate of CD4+ depleted animals consisted almost completely of CD8+T cells and NK cells. This result suggests that CD4+ T cells could inhibit the tumour infiltration by CD8+ T cells (Martinotti et al., 1995).

In the case of MCA 207 and MCA 102 sarcomas, which are differentially immunogenic, IL-12 secretion by tumour cells led to tumour eradication and resistance to rechallenge with original tumour cells. IL-12 engineered cells also protected against

distant site inoculation with original tumour cells. NK cells and IFNγ were crucial in the early phase of the anti-tumour response, and both CD4+ and CD8+ T cells were involved in the following phase that led to long-term immunity (Tahara et al., 1995).

Further preclinical studies in mice indicated that establishment of a BL-6 tumour, that is a poorly immunogenic melanoma, was delayed significantly when tumour cells were admixed with IL-12 transduced fibroblasts. In addition, preimmunisation with IL-12 secreting fibroblasts together with irradiated BL-6 cells significantly delayed the growth of a subsequent BL-6 challenge. Histological examination revealed that the regression of BL-6 is associated with peritumoural accumulation of macrophages and decreased numbers of CD4+ T lymphocytes in the BL-6 tumour (Tahara et al., 1994).

iv) IFN-γ gene

Immunisation with high IFN-γ-secreting engineered tumour cells of mice carrying established micro-metastases resulted in almost complete cure of mice by inducing cytotoxic T lymphocytes (Porgador et al., 1993).

IFN-γ expression in the malignant mouse C1300 neuroblastoma (Watanabe et al., 1989) resulted in an augmented class I MHC antigen expression and induced an improved specific anti-tumour immunity in the host. Other studies in several types of tumour cell lines derived from various mouse strains, like CMS-5 fibrosarcoma (Gansbacher et al., 1990), SC115 mammary carcinoma tumour (Teramura et al., 1993), J558L plasmacytoma (Hock et al., 1993b) and LP92-T lung carcinoma (Yanagihara et al., 1994), also report that IFN-γ- locally secreted by engineered tumour cells can lead to tumour rejection in T cell competent-, but not nude, mice. By contrast, studies on some models, including MCA101 fibrosarcoma (Restifo et al., 1992), B16 melanoma (Dranoff et al., 1993) and OVHM13 ovarian tumours (Yanagihara et al., 1994), did not reported a suppression of tumour by IFN-γ transduced tumour cells.

The mechanism of rejection by syngeneic hosts involved a specific CD8+ T cell-dependent immunity (Gansbacher et al., 1990; Restifo et al., 1992). The reduced tumour growth theoretically can result from an enhancement of MHC molecule expression, induced by IFN-γ, on tumour cells and on professional antigen presenting cells (Restifo et al., 1992), but it is likely that the activation effect of IFNγ on CTLs (Klavinskis et al., 1989), macrophages and NK cells (Herberman et al., 1982) also contributes to the immune reaction.

Human melanoma cells, transduced with the IFN-γ gene by retroviral transfection, have been obtained that secret biologically active IFN-γ and exhibit enhanced expression of MHC class I and class II molecules. In addition, lymphocytes stimulated with IFN-γ secreting melanoma cells revealed increased lytic activity against the autologous tumour (Abdel-Wahab et al., 1994).

v) Other cytokine genes

Systemic anti-tumour immune responses have been demonstrated in J558 plasmacytoma cells engineered to secrete IL-7. Immunity was independent of CD8+ T cells, but dependent on CD4+ T cells and macrophages (Hock et al., 1991a). Tumour rejection was observed only in syngeneic animals, but not in T cell deficient mice, demonstrating a complete T cell dependence (Hock et al., 1991a; McBride et al., 1992).

An anti-tumour effect by transduction of the IL-7 gene was also reported in a murine fibrosarcoma and in a glioblastoma model, in which the effector cells responsible for tumour suppression were CD8+ T cells (Aoki et al., 1992; Jicha et al., 1991). The variable T cells subsets required for tumour rejection in the different models may reflect the particular level of MHC class I or II molecules expressed by the respective tumour cells.

Introduction of the TNF-α gene into tumour cells was studied in different mouse tumour models (Asher et al., 1991; Karp et al., 1993; Budillon et al., 1991). TNF-α-transduced tumour cells typically grew more slowly *in vitro* and were found to regress after an initial phase of growth in a dose-dependent manner. This tumour regression was abrogated by the depletion of CD4+ and CD8+ subsets *in vivo*, suggesting that T cells are involved in the immune response (Asher et al., 1991).

In a separate study, macrophages were observed at the sites of a murine plasmacytoma that was engineered to secrete TNF-α, suggesting the implication of inflammatory cells in the rejection of this tumour (Blankenstein et al., 1991).

Granulocyte macrophage colony-stimulating factor (GM-CSF) is known to increase the number of granulocytes, eosinophils and monocytes *in vivo* and to be crucial for the differentiation and survival of dendritic cells (Steinman, 1991).

In an elegant study, which compared the ability of ten different molecules to enhance the immunogenicity of tumour cells in a murine melanoma model, GM-CSF appeared to be the most effective in producing potent, long-lasting and specific anti-tumour immunity requiring both $CD4^+$ and $CD8^+$ cells (Dranoff et al., 1993). GM-CSF is supposed to recruit dendritic cells that pick up and present tumour-specific antigens to T cells, thus inducing specific immunity and memory (Huang et al., 1994).

An interesting study has recently evaluated an approach based on expression of IL-6 and its receptor in tumour cells. IL-6 exerts its activity through a membrane-bound receptor complex consisting of alpha (gp-80) and beta-subunits (gp-130). IL-6 first binds gp-80, the complex attracting two gp-130 molecules which leads to signal transduction. The effect of IL-6 may be enhanced by its agonistic soluble alpha-receptor subunit (sIL-6R) (Mackiewicz et al., 1992; Mackiewicz et al., 1995). The IL-6/sIL-6R complex *in vivo* may also activate cells lacking the alpha-subunit, but possessing only

the beta-subunit of the receptor. Most of the immune cells express beta, but not alpha-subunit of the IL-6 receptor and sIL-6R genes (Mackiewicz et al., 1995).

In a mouse model, the immunisation of mice with B78H1 melanoma cells modified with IL6/sIL-6R genes stimulated long lasting anti-melanoma immunity, through activation of CD8+ T cells and NK cells (Mackiewicz et al., 1995).

Several clinical studies using either autologous or allogeneic cytokine gene-transduced tumour cells or tumour cells plus transfected bystander cells have been initiated. Only preliminary results of phase I/II clinical trials have been published. They show minimal toxicity of immunotherapy with genetically modified tumour cells and the induction of a specific immunity against tumour antigens. However, the earliest available results show that only about 10% of patients displayed an objective response (Abdel-Wahab et al., 1997; Simons et al., 1997; Belli et al., 1997; Parmiani et al., 1996; Roth et al., 1997).

1.4.3.2 Genetic modification of APC cells

Gene transfer techniques also have been employed to improve antigen presenting cells activity, by transduction of tumour-specific antigens.

Genes encoding the specific antigens are transfected *in vitro*, or *in vivo* delivery has been used to transduce antigen presenting cells by different strategies, including the use of recombinant viral and bacterial vaccines, or DNA plasmid injection.

The intrinsic immunogenicity of viruses together with the development of standard techniques to engineer recombinant viruses has engendered broad interest in recombinant viral vaccines. Two mechanisms underlie the capacity of recombinant viral vaccine to initiate immune responses. First, the cellular damage induced by viral

infection elicits danger signals that attract and activate bone marrow derived APCs. This activation results in presentation of the transduced tumour antigen in the context of costimulatory molecules. The second mechanism is based on the ability of some viruses to directly infect bone marrow derived APCs. In this way, viral transduced genes encoding tumour antigens are endogenously synthesised in the APC cells and efficiently processed and presented in the MHC class I pathway.

The capacity of some viruses to directly infect APCs allows for modification of recombinant viral vaccines to enhance the processing and presentation of encoded antigens. For example, graft of endosomal/lysosomal sorting signals onto the gene encoding antigen, cloned in recombinant poxviruses, has been reported to enhance MHC class II processing by antigen presenting cells and CD4+ T cell activation (Lotze et al., 1996; Tepper et al., 1994).

Vaccination with recombinant viral vectors expressing melanoma antigens (Bronte et al., 1997; Wang et al., 1995), differentiation antigens CEA (Abrams et al., 1997) or MUC-1 (Akagi et al., 1997) have been described. These vaccinations resulted in a specific anti-tumour immunity and tumour regression.

A number of bacterial strains, including Salmonella (Abdel-Wahab et al., 1997; Wolff et al., 1990; Condon et al., 1996) and Listeria monocytogenes (Pardoll et al., 1995; Doe et al., 1996), infect monocytes and macrophages and can therefore target antigens to professional APCs.

Recombinant Listeria vaccines have been tested in animal models of macroscopic cancer with promising results (Corr et al., 1996; McCabe et al., 1995). The peculiar two-phase intracellular life cycle of this bacterium (Falkow et al., 1992), which, upon infecting monocytes and macrophages, is characterised by the presence of a phagolysosomal phase and a cytolytic phase, resulted in delivery of transduced antigens to both class I and class II pathways.

Some data have demonstrated the efficacy of this strategy in regression of established macrometastases (Pan et al., 1995).

1.4.3.3 Genetic modification of T cells

Strategies to enhance the therapeutic efficacy of tumour-infiltrated lymphocytes (TILs) through genetic modification have been explored. However, limited success was obtained, due to the inefficiency of gene transfection and *in vivo* tumour targeting. Moreover, the use of this approach in a clinical setting is limited by the difficulty of generating large numbers of tumour-specific T cells at the single patient level without using high amounts of cytokines, which may cause apoptosis or expansion of nonspecific T cells.

In one reported approach, autologous T cells have been transfected with a chimeric T cell receptor bearing the tumour-specific binding domain of a monoclonal antibody to enhance their recognition and killing of tumour cells (Gross et al., 1992). Gene modification of effector cells with these chimeric receptors offers the advantages of a permanent association between receptor and effector cell and the retention of chimeric receptor expression in daughter cells following cell proliferation.

For example, it has been shown recently that nude mice xenotransplanted with ovarian cancer cells can be cured by injection of autologous T cells bearing a chimeric receptor with the ScFv of an antifolate receptor highly expressed by ovarian cancer cells (Hwu et al., 1995).

In another approach, tumour infiltrating lymphocytes have been transduced with genes encoding T-cell stimulatory cytokines to activate or enhance their anti-tumour activity. For example, TIL retrovirally transduced with the TNFα gene have been generated in

an attempt to deliver high concentrations of TNF to tumour sites without inducing systemic toxicity (Rosenberg, 1992).

1.4.4 OTHERS GENE THERAPY APPROACHES

1.4.4.1 Chemo-gene therapy

The introduction into tumour cells of suicide genes encoding enzymes that convert nontoxic substances (prodrugs) into toxic metabolities is an attempt to avoid the systemic side-effects of conventional chemotherapy. The main task is to target the genes specifically to the tumour cells and to reach as many tumour cells as possible. Recent reports provide increasing evidence for the so-called "bystander effect", the cytolysis of nontransduced tumour cells, involving both cell-to-cell transfer of the active metabolites and stimulation of immune-mediated responses against the tumour cells (Freeman et al., 1995).

Moolten (Moolten, 1986) initially reported that tumour cells could be rendered ganciclovir-sensitive by herpes simplex virus-thymidine kinase (HSV-tk) gene transfer and that mice injected with transfected tumour cells could be cured with systemic ganciclovir treatment. HSV-tk phosphorylates ganciclovir approximately 1000-fold more efficiently than mammalian thymidine kinase. The resulting ganciclovir monophosphate is further phosphorylated by the mammalian enzyme to ganciclovir diphosphate and triphosphate, which are cytotoxic because they inhibit DNA polymerase (Davidson et al., 1981; Elion et al., 1977). Culver (Culver et al., 1992) pioneered the use of HSV-tk gene therapy for cancer showing complete macroscopic and microscopic regression of gliomas in ganciclovir-treated rats which were injected intratumourally with murine fibroblasts transduced with an HSV-tk retroviral vector.

After these preliminary results, a number of studies have been developed using different approaches.

For example, a combination of HSV-tk-IL-2 gene therapy was evaluated in mice injected with the MCA26 colon cancer, using two adenoviral vectors containing HSV-tk and murine interleukin-2 genes (Chen et al., 1995). Following ganciclovir treatment, a massive tumour necrosis surrounded by inflammatory cells, was present and a significantly enhanced anti-tumour reactivity was observed in animals treated with a combination of the two vectors. The use of FLC lines expressing both HSV-tk and interferon-α followed by ganciclovir treatment resulted in cure of 70% to 100% of mice, which were also resistant to a subsequent challenge of wild type parental tumour cells (Santodonato et al., 1996).

In another approach, a targeting of the suicide gene expression specifically in murine melanocytes and melanoma cells was obtained using the murine tyrosinase promoter to express the HSV-tk gene. Mice intravenously injected with retroviral supernatant transducing the gene showed a dramatic reduction in the number of B16 melanoma lung metastases after ganciclovir treatment (Vile et al., 1994). Promoters for the human alpha-fetoprotein gene and carcinoembryonic antigen gene have also been used to direct HSV-tk expression to hepatoma (Ido et al., 1995) or gastric cancer cells (Tanaka et al., 1996), respectively.

Cytosine deaminase (CD) has also been used as a suicide-gene. The product of this gene acts by converting nontoxic 5-fluorocytosine to toxic 5-fluorouracil by deamination. 5-fluorocytosine is converted to 5-fluorouracil monophosphate and to 5-fluorouracil triphosphate, which block thymidylate synthetase and mRNA transcription, respectively.

The transfection of a weakly immunogenic colon cancer and C57BL/6 mice-derived fibrosarcoma cell lines with the cytosine deaminase gene has been reported (Mullen et al., 1994). A significant tumour inhibition was observed after treatment with systemic 5-fluorocytosine, when these cells were subcutaneously injected into syngeneic mice. A significant reduction in the number of secondary tumours was also detected upon rechallenge with syngeneic wild-type tumours.

1.4.4.2 Introduction of tumour suppressor genes

The transfer of tumour suppressor genes, including p53, p21 and the retinoblastoma gene (Rb), into tumour cells has been studied as a method to overcome the loss of cell cycle control and to block tumour cell growth. Multiple human and murine tumour cell lines have been exploited for *in vitro* studies, as well as *in vivo* murine tumour models. These studies demonstrated reduced tumour cell proliferation and suppression of tumour formation in mice.

The p53 gene is central to both the cell cycle and differentiation and plays a pivotal role in apoptosis. Upon DNA damage, the level of p53 is increased, ultimately leading to cell-cycle arrest in G1 to allow damage repair. P53 acts as a transcription factor to activate or repress transcription by binding to p53 target motifs in various promoters.
In a p53-deficient human prostatic tumour cell line, adenovirus-mediated transfer of wild type p53 gene has been reported to produce high levels of wild-type p53 protein, which lead to apoptosis, and failed to form tumours in nude mice (Yang et al., 1995). Replication-deficient adenovirus transfer of wild type p53 gene has also been used in squamous cell carcinoma of the head and neck (Clayman et al., 1995), primary and metastatic murine bladder tumour cells (Werthman et al., 1996), murine and human melanoma cell lines (Cirielli et al., 1995), the ovarian cancer cell line 2774 (Santoso et al., 1995), human T987G glioma cells and H23 small-cell lung carcinoma (Gjerset et

al., 1995) and the 9L glioma cell line (Badie et al., 1995), with *in vitro* or *in vivo* growth suppression of infected cells via p53-mediated apoptosis.

A retrovirus-mediated wild type p53 gene transfer into MDAM 231 breast cancer cells, that contain endogenous mutant p53, resulted in suppression of tumour formation in nude mice (Runnebaum et al., 1995). Furthermore, transduced human medulloblastoma cells, which endogenously express mutant p53, with a herpes simplex virus vector expressing wild type p53 resulted in cell-cycle arrest (Rosenfeld et al., 1995).

A method to achieve systemic therapy with wild type p53 was evaluated using a liposome-p53 complex, that resulted in significant tumour regression, injected into nude mice inoculated with breast carcinoma cells (Lesoon-Wood et al., 1995).

The product of the p21 gene binds proliferating cell nuclear antigen to prevent DNA replication; it also acts as a cyclin-dependent kinase inhibitor, preventing cdk-mediated phosphorylation of Rb. Overexpression of p21 is known to lead to cell cycle arrest at the G1/S boundary, making it an excellent candidate for cancer gene therapy. Eastham (Eastham et al., 1995) transferred the p21 gene under the control of the cytomegalovirus promoter into p53-deficient mouse prostate cancer cells via an adenoviral vector. The infected cells showed overexpression of p21 protein and downregulation of the cell-cycle-dependent kinase activity.

The retinoblastoma gene was the first tumour-suppressor gene identified and has been shown to play a role in suppression of tumour invasiveness (Zhou et al., 1994), antiangiogenesis (Richardson et al., 1995) and immunogenicity of tumour (Lu et al., 1994; Li et al., 1994). Unphosphorylated retinoblastoma is able to sequester transcription factors that regulate cell proliferation, keeping the cell in a quiescent state.

A constitutively active N-terminal truncated retinoblastoma protein (94 kDa) was constructed and has been characterised as possessing slower turnover and less

phosphorylation than the wild type retinoblastoma protein (110 kDa). Replication-deficient adenoviral-mediated gene transfer of the truncated retinoblastoma gene into nude mice bearing either human non-small-cell lung carcinoma or bladder carcinoma rendered more potent tumour suppression than transfer of the wild-type retinoblastoma gene (Xu et al., 1996).

1.4.4.3 Inhibition of angiogenesis

Angiogenesis is a crucial requirement for tumour growth and its inhibition appears to be a promising strategy for cancer treatment. Anti-angiogenesis gene-therapy has been evaluated by the use of different approaches (Kong et al., 1998).

Gene therapy can be used to antagonise stimulators and to enhance inhibitory factors. Tumour-derived vascular endothelial growth factor (VEGF) has been shown to be a major inducer of tumour vascularization. VEGF is an endothelial-specific mitogen, which acts by specific interaction with two transmembrane tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2).

Transfection with antisense constructs to VEGF appeared to reduce tumourigenicity of glioma cells in nude mice (Saleh et al., 1996). A retroviral vector was used to infect endothelial cells with a dominant negative mutant gene encoding the VEGF receptor-1 (VEGFR-1). This mutant receptor appeared to dimerize with native VEGFR-1 and to prevent signal transduction and endothelial cell activation with VEGF (Millauer et al., 1996). Furthermore, retroviruses encoding a truncated VEGF receptor 2 (VEGF-2) have been reported to suppress tumour angiogenesis and prolong survival time of rats bearing syngeneic intracerebral glioma tumours. A dual mode of function of truncated VEGFR-2, consisting in a dominant-negative inhibition of wild-type VEGFR-2 function and VEGF depletion by receptor binding, was proposed as mechanism involved in angiogenesis inhibition (Machein et al., 1999).

The use of transgene products that specifically target tumour endothelial cells is another approach. *In vitro* studies have evaluated the use of promoter sequences from genes that are expressed preferentially on endothelial cells, such as VEGFR-2 and E-selectin, to express the transgene. These promoter sequences may allow selective expression of endothelial inhibitors or toxins to cause vascular destruction (Ebeling et al., 1993).

1.5 BACTERIA AND CANCER

There is considerable historical evidence concerning the antagonisms between acute bacterial infections, or their toxins, and cancer.

Beginning from the 18th century, remarkable cases of cancer which had recovered following an acute bacterial infection have been reported. These cases were often called "spontaneous regression" and some of these occurred following acute inflammation or fever (Nauts, 1980).

In the 1890s, William Coley, the first tumour immunologist, began to treat cancer patients with preparations derived from streptococcal cultures (Coley's toxins) to activate general systemic immunity, a portion of which might be directed against the tumour (Nauts, 1989). Studies at that time also pointed to a lower frequency of cancer in patients with tuberculosis.

Beginning from 1970 mycobacteria, in particular *Mycobacterium bovis* (bacillus Calmette Guerin), have been used in experimental model systems and in humans as an approach to activate a specific and/or non-specific anti-cancer immunity though this has been with variable results.

1.5.1 IMMUNITY TO MYCOBACTERIA

Mycobacteria were the first bacterial pathogens of man to be described and they are responsible for more mortality than any other pathogen, particularly in developing countries.

They are slow-growing, aerobic, facultative intracellular bacilli. The two common human pathogens in this class of bacteria are *M. tuberculosis* and *M. leprae*; in addition,

atypical mycobacteria such as *M. avium* cause opportunistic infections in immunodeficient hosts, in particular AIDS patients. *M. bovis* infects cattle and may infect humans; bacillus Calmette Guerin (BCG) is an attenuated, nonvirulent strain of *M. bovis* that is used as a vaccine against tuberculosis.

Mycobacterium tuberculosis causes a respiratory infection, which may be asymptomatic in some individuals or may produce damaging pulmonary lesions with severe debilitation in others. If the infection is not restricted by immune responses it can disseminate to the major organs of the body, most frequently in a fatal form.

Given the seriousness of the disease, research involving immunity to *M. tuberculosis* is most prominent among this class of intracellular bacterial parasites. Current efforts are focused on the development of effective vaccines and much of the work is directed towards elucidation of the mechanism of protective immunity.

1.5.1.1 Mechanisms involved in protective immunity

Classical studies have demonstrated that immunity to *Mycobacterium tuberculosis* is mediated by cells, whereas humoral responses were found not to be of importance (Andersen, 1997).

Much of the work performed in the mouse model has, collectively, tended to support the hypothesis that cells mediating specific resistance to mycobacterial infection fall into the category of T helper type 1 (Th1) $CD4^+$ cells, based upon their pattern of cytokine secretion. Indeed, kinetic studies performed in mice revealed the emergence of $CD4^+$ cells, and to a lesser extent $CD8^+$ cells, in response to intravenous infection with live, virulent *M. tuberculosis* that secreted IFN-γ (Orme et al., 1992a). The emergence of these cell populations coincided with the initial containment of the progressively growing bacterial infection and was then followed, a few weeks later, by the emergence of a second population of $CD4^+$ cells that had strong cytolytic activity. Similarly, in two

studies using bacillus Calmette Guérin (BCG), the secretion of IFN-γ correlated strongly with the expression of protective immunity (Kawamura et al., 1992; Huygen et al., 1992). Interleukin-12 is of crucial importance for murine Th1 cell development (Hsieh et al., 1993); the main source of IL-12 during infection is the macrophages which are stimulated by the phagocytic event (Fulton et al., 1996).

In a study in humans (Barnes et al., 1993), a slightly different picture has emerged: the majority of T cell clones generated from patients with *M tuberculosis* infection tended to make a broad spectrum of cytokines, including IFN-γ, tumour necrosis factor (TNF)-a, and interleukin (IL)-10.

In addition to a response involving αβ T-cells, some studies have shown that both *in vivo* and *in vitro M. tuberculosis* stimulates the minor subsets of T cells expressing the γδ form of the antigen receptor, which are $CD4^-$, $CD8^-$ and $CD3^+$.

γδ T cells accumulate in relatively large numbers in mice infected with *M tuberculosis* (Griffin et al., 1991) and evidence for strong recognition *in vivo* of a 16 amino acid peptide sequence within the mycobacterial heat shock protein (HSP) 60 was obtained (Fu et al., 1993). It is postulated that the response of γδ T cells to heat shock proteins is a primitive defence mechanism against some microbes, which are involved as a first line of defence.

With regard to γδ T cells obtained from human patients, however, the HSP 60 molecule does not appear to be a primary target of immunity (Boom et al., 1992). Also in humans, such cells respond strongly to live tuberculosis infection and the degree of accumulation/expansion of γδ T cells in infectious lesions seems to relate directly to the degree of protection that subsequently ensues (Barnes et al., 1992). The biological role of lymphocytes bearing γδ T cells is still unclear. Some data support the idea that γδ T cells may represent a profuse source of cytokines, since IFN-γ and IL-2 are secreted by

γδ T cells *in vitro* after activation with bacteria (Barnes et al., 1992; Follows et al., 1992).

A third immune population appeared to be involved in response to mycobacteria. This population, represented in very low percentages, is $CD4^-$, $CD8^-$ but $\alpha\beta$ TCR^+ T cells. It has been demonstrated that this cell type recognises mycolic acid from *M. tuberculosis*, presented by the non-MHC encoded CD1 molecule (Beckman et al., 1994). Indeed, CD1 proteins have been proposed as antigen-presenting molecules that have evolved the ability to present nonpeptide lipid antigens to T cells.

CD1-restricted T cells have been derived from the tissue lesions and blood of patients with mycobacterial infections. This cell population has been proposed to contribute to host defence by mediating the lysis of infected macrophages by two different mechanisms. First, a direct killing of infected macrophages, supported by *in vitro* experiments which shows a high degree of cytolytic activity against antigen-pulsed CD1+ mononuclear phagocytes (Stenger et al., 1997; Moody et al., 1999) and recognition and lysis of CD1+ targets infected with live virulent M tuberculosis bacilli (Stenger et al., 1997; Jackman et al., 1998). Second, an indirect effect based on the recruitment of freshly macrophages to destroy infected cells, which is suggested by high levels of interferon γ and low levels of IL-4 (Gong et al., 1998), typical of the Th1 pattern of cytokines required for activation of macrophage-mediated killing of intracellular pathogens (Sieling et al., 1995), released *in vitro* by mycobacteria-specific CD1 restricted T cells.

1.5.1.2 *Mycobacterium tuberculosis* antigens

Using a clever fractionation method to separate proteins by molecular size, initial progress has been made in defining more precisely the key proteins involved both in

protective (Andersen et al., 1991; Andersen et al., 1992) and memory immune responses (Andersen et al., 1993).

A consistent number of antigens have been identified and some of these proteins have been classified as "immunodominant" on the basis of their ability to induce antibody, or proliferative T-cell responses in humans and experimental animals. Generally, the immunogenicity of these antigens may be explained on the basis of two different properties (Young et al., 1991):

- several antigens belong to highly conserved stress protein families (Heat Shock Protein) (Young et al., 1988). Heat-shock proteins are molecular chaperones, mediating the assembly and folding of other proteins. They are among the most immunogenic molecules known (Kaufmann, 1990; Cohen et al., 1991; Young et al., 1988). The cause of this immunodominance is thought to reside in their universal distribution and high degree of sequence conservation, resulting in continous priming of the immune system.
- Several antigens are secreted or released proteins of *M. tuberculosis*, which may be recovered in the culture filtrate (Harboe et al., 1986; Abou-Zeid et al., 1988). It has been proposed that proteins which are secreted from intact, viable mycobacteria may have a special role in protective immune mechanism, since they are available for immune recognition at an early stage of infection. Live dividing mycobacteria are, indeed, more effective than killed bacilli in evoking protective immunity (Collins et al., 1988; Orme, 1988) and only T cells recognising peptides secreted from live bacteria during the early phase of infection provide efficient immune surveillance and protection. Therefore, these proteins represent an important target of a strong cellular response (Andersen et al., 1991; Andersen et al., 1992; Andersen et al., 1993).

Based on studies of protein release during growth of M tuberculosis, three different groups have been defined: excreted proteins, secreted proteins of the outer cell wall and cytoplasmic proteins released at late culture timepoints. A short term culture filtrate (ST-CF) containing a mixture of shedded outer cell wall proteins, excreted proteins and a minimal content of autolytic products, released during the first phase of growth, has been defined. This filtrate has been evaluated particularly for antigenic properties in experimental vaccines against tuberculosis (Andersen, 1994; Andersen et al., 1992; Andersen, 1997). Antigens of *Mycobacterium tuberculosis* contained in this short term culture filtrate (ST-CF) have been purified and characterised (Andersen, 1997) (Fig. 1.15).

Dna K (70 kDa) and GroES (10 kDa). These molecules are heat shock proteins, which function intracellularly as molecular chaperones (Young et al., 1991). They are widely conserved and can be found in various procaryotes and eukaryotes. In heat-shocked eukaryotic cells, DnaK has been demonstrated on the cell surface as well as in the cytoplasm (Rocchi et al., 1993).

Pro rich complex (45-47 kDa). This antigen initially was identified in *M. bovis* BCG culture filtrates as a target for both delayed type hypersensitivity (DTH) and antibody responses after immunisation with live, as opposed to dead, bacteria (Romain et al., 1993; Romain et al., 1993). The corresponding gene from M tuberculosis was sequenced and found to encode a 39 amino acid consensus signal peptide (Laqueyrerie et al., 1995). The molecule has a predicted molecular mass of 29 kDa, but a high percentage of proline (21.7%) gives an increased rigidity and changes the mobility of the molecule and the apparent molecular mass.

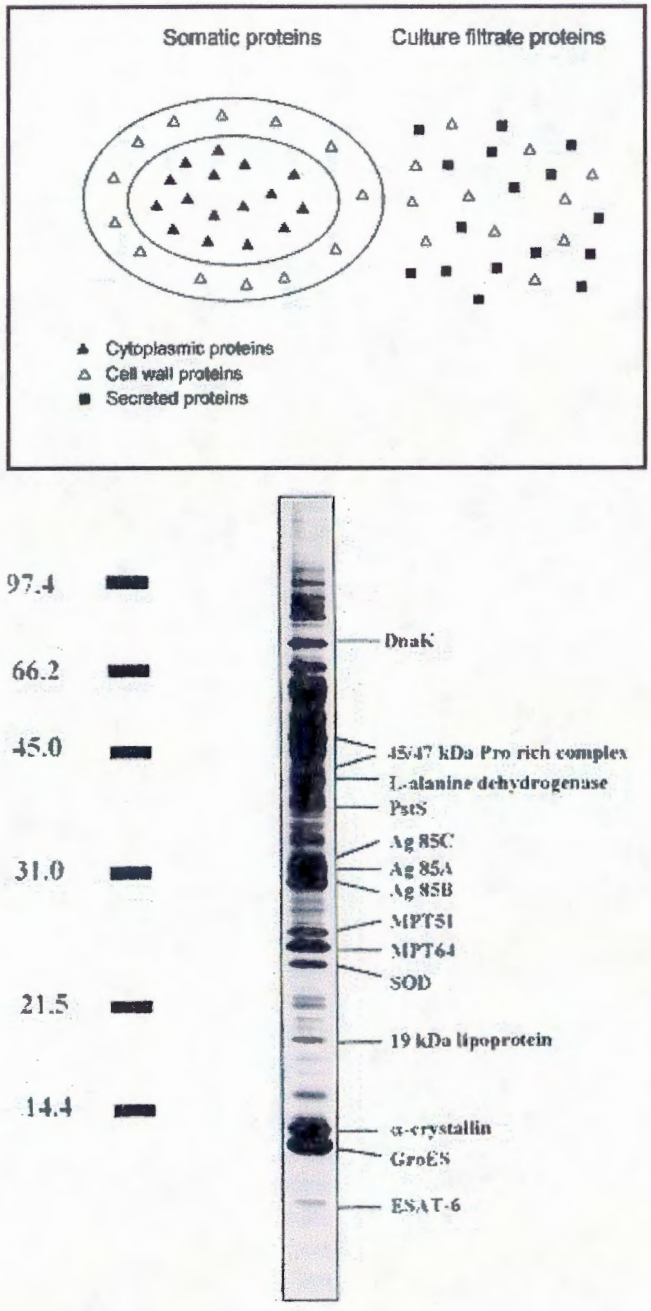


Fig. 1.15: Mycobacterium tuberculosis antigens

Proteins from M. tuberculosis can be divided into three major classes depending of their subcellular localization. ST-CF will consist of a mixture of secreted proteins and shedded outer cell wall proteins.

The composition of a defined ST-CF harvested fromalogarithmically growing culture of M. tuberculosis: the culture filtrate has been separated in SDS-PAGE and silver stained, the localization in ST-CF of previously characterized proteins has been indicated (from Andersen et al., 1997).

L-alanine dehydrogenase (40 kDa). This enzyme catalyses the reversible conversion of pyruvate to L-alanine and may be involved in cell wall synthesis, as L-alanine is one of the four amino acids constituting the peptide moiety of the peptidoglycan layer (Young et al., 1991).

Phosphate binding protein Ag38 (38 kDa). This molecule is localised mainly in the outer cell wall and released to the surroundings in limited amounts. The protein is post-translationally modified by lipidation and apparently is anchored in the cell membrane by its lipid moiety (Young et al., 1991). The function of the 38 kDa protein is to bind and make phosphate available to the bacteria and the synthesis of this protein is highly up-regulated during phosphate starvation (Andersen et al., 1990). Immunogold labelling of bacteria grown in medium without phosphate demonstrated the antigen mainly in the cell wall and on the cell surface (Espitia et al., 1992). A signal sequence was identified in the gene encoding this protein (Andersen et al., 1989).

Several studies have demonstrated that the 38 kDa protein is one of the most important antigens of *Mycobacterium tuberculosis*, able to induce both serological and cell-mediated reactivity (Kadival et al., 1987; Harboe et al., 1992; Young et al., 1986). Mice sensitised against the 38 kDa antigen preferentially appeared to induce a Th1-like immune response, revealed by secretion of IL-2 and IFN-γ and the preferential production of IgG2a-type antibody (Agrewala et al., 1995).

AG 85 complex (30-32 kDa). This group of outer cell wall antigens consists of three distinct, although markedly cross-reacting, components denoted AG85A, B and C (Wiker et al., 1992). All three components demonstrate varying degrees of fibronectin binding and these molecules have been suggested to play an important role in macrophage uptake of mycobacteria (Abou-Zeid et al., 1991). Some data indicate that AG85 holds mycolyl-transferase activity indicating that outer cell wall synthesis may be

the primary function of this molecule (Belisle, 1995). The three components are encoded by three separate genes (Wiker et al., 1990; Content et al., 1991). Signal sequences have been identified.

The Ag85 complex has been demonstrated to induce strong T-cell proliferation, interferon-γ production and cytotoxic T lymphocyte (CTL) activity in most healthy individuals infected with *M. tuberculosis* (Launois et al., 1994). Furthermore, immunisation of mice with plasmid DNA constructs encoding the antigen 85 induced substantial humoral and cell-mediated immune responses and conferred significant protection against challenge with live *M. tuberculosis* (Huygen et al., 1996).

MPT51 (27 kDa) and MPT64 (26 kDa). Both proteins are major components of short term culture filtrate (ST-CF). MPT51 and the AG85 complex contain cross-reactive B cell epitopes, and sequence homology between the AG85 components and MPT64 has been demonstrated (Wiker et al., 1992). These findings suggest the existence of secreted antigens with common structural features. No information about the possible functional relevance of these molecules presently is available. MPT64 is so far the only secreted antigen with no intermediate position in the cell wall (Andersen et al., 1991).

Superoxide dismutase (23 kDa). This molecule exists as a tetramer in its native form and has an estimated molecular mass of 88 kDa and an apparent subunit molecular mass of 23 kDa (Andersen et al., 1991; Kusunose et al., 1976). The protein is found among the proteins appearing very early in culture filtrates (Andersen et al., 1991). The molecule may play an important role in protecting bacteria from the toxic effect of superoxide radicals generated during the oxidative burst in the macrophage and can therefore be regarded as a virulence factor.

19 kDa lipoprotein. This molecule is secreted across the cell membrane and is anchored in the outer cell wall by a lipid moiety. The molecule appears to be post-translationally glycosylated (Fifis et al., 1991; Garbe et al., 1993) and the gene encodes a consensus signal peptide (Ashbridge et al., 1989).

Alpha-crystalin (12/16 kDa). This *M. tuberculosis* homologue of the alpha-crystalin family of low molecular mass heat shock proteins possesses sequence identity with members of the family of small heat shock proteins which includes the 18 kDa antigen from *M. leprae* (Verbon et al., 1992). The molecule exists predominantly as a 12 kDa molecule in culture filtrates whereas the form present in mycobacterial extracts has an apparent molecular mass of about 16 kDa.

ESAT-6 (6 kDa). A low mass secreted protein, identified due to its high reactivity with T cells isolated from memory immune mice (Andersen et al., 1995). Importantly, the gene encoding ESAT-6 is lacking in all strains of BCG tested whereas it is found in virulent *M. bovis* (Harboe et al., 1996). In agreement with this finding ESAT-6 recently was identified in a segment deleted in all BCG substrains believed to be the original mutation leading to attenuation of *M. bovis* (Mahairas et al., 1996).

1.5.1.3 Pathogenesis of tuberculosis

Tuberculosis is an example of infection with an intracellular bacterium in which protective immunity and pathologic hypersensitivity co-exist, and the lesions are caused mainly by the host response.

Mycobacterium tuberculosis is inhaled as droplet nuclei, each containing one to three bacteria. These small particles are carried via the airstream and distributed to all regions of the lung (Wiegeshaus et al., 1989). The mycobacteria are taken up by alveolar macrophages; the bacteria may either be destroyed or start to multiply after a lag period

of a few days. Pro-inflammatory cytokines and chemokines secreted from infected macrophages lead to the recruitment of monocytes and lymphocytes from the blood and the development of the inflammatory process (Davatelis et al., 1988; Taub et al., 1993; Taub et al., 1993; Murphy et al., 1996). After about 2 weeks of infection (Orme, 1987; Andersen et al., 1992) the first signs of specific immunity emerge and involve the triggering of cytokine release from specific T lymphocytes (Andersen et al., 1992; Orme et al., 1993b; Andersen et al., 1995). The cytokines activate the bacteriostatic activity of macrophages and accelerate the lymphocyte recruitment. As the process progresses, chronic antigenic stimulation leads to maturation of monocytes into epithelioid cells and multi-nucleated giant cells surrounded by T lymphocytes, resulting in the formation of granulomas surrounding the microbes (Turk et al., 1982). The outcome of disease is determined by a dynamic balance between host and parasite factors. In the resistant individual a high level activity may be reached rapidly and the disease consequently is controlled in the asymptomatic stage. In the susceptible host, by contrast, the process of bacterial multiplication and cellular recruitment continues, the primary lesion enlarges and some bacteria are transported to the regional lymph nodes, giving rise to a granulomatous reaction. As the disease continues, the amplified immune reaction leads to intense inflammation, tissue destruction, caseous necrosis and the formation of cavitary lesions (Dannenberg, 1991).

1.5.2 USE OF BCG IN CANCER IMMUNOTHERAPY

The first published use of BCG as a cancer vaccine was in 1935 by Holmgren in Sweden but it was not until the late 1950s and 1960s that experimental and clinical studies generated enthusiasm for its use against various cancers, including leukaemia, colon cancer, lung cancer and melanoma. In particular, the most influential report was that of Mathe in 1969 for the treatment of lymphoblastoid leukaemia (Mathe et al., 1969). However, failure of later controlled studies to confirm these results, plus the advent of modern chemotherapy and radiotherapy, led to gradual abandonment of the use of BCG for most cancers.

However, BCG immunotherapy has been demonstrated to be effective specifically in bladder cancer treatment. Studies on the use of BCG in bladder cancer revealed that the bladder was capable of mounting a strong immune response and that close contact between BCG and the cancer was required for maximum effect in animal models (Crispen, 1989; van der Meijden et al., 1989). In 1976, Morales described the successful treatment, with intravesical instillation of BCG, of a small group of bladder cancer patients (Morales et al., 1976). Wide acceptance of BCG immunotherapy for bladder cancer, however, began after a controlled study in 1980 that showed the unequivocal benefits in terms of decreased recurrence rate and increased median time to recurrence in patients given BCG immunoprophylaxis after local surgery (Lamm et al., 1980).

At present, mostly patients with superficial bladder cancer are treated successfully with intravesical instillation of BCG and this treatment has been compared in several trials with all other forms of intravesical chemotherapy. BCG in general has proved more effective than chemotherapy, especially when trials have been weighted towards more aggressive high-grade disease and where re-induction and maintenance therapy has been given (Lamm et al., 1995; van der Meijden et al., 1989; Herr, 1992). In addition to its

role as an immunoprophylactic agent, BCG has also shown a 50-60% effectiveness against small residual tumours and a 70-75% complete response rate for carcinoma-in-situ. BCG is not effective for muscle-invasive disease, or for tumours that lie out of direct contact with BCG, such as those deep within the prostate or in the upper urinary tract.

The use of BCG for bladder cancer does not come without drawbacks. First, the response to BCG is unpredictable (Herr, 1992). There are currently no reliable prognostic factors that accurately predict treatment success or failure.

Second, BCG has side-effects (Lamm et al., 1989). Most patients experience local symptoms of cystitis including frequency, urgency, dysuria and occasional haematuria. Mild systemic symptoms of high temperature, malaise and a transient influenza-like illness are also common. Severe side-effects occur in 5% of patients, roughly 10% of which involve frank BCG sepsis. Seven deaths due to BCG sepsis from bladder instillations have been documented.

Whether the anti-cancer response is specific is probably one of the most debated questions in relation to BCG immunotherapy of bladder cancer. The simplest explanation is that the intravesical instillation of BCG results in non-specific cystitis, which is accompained by the local production of cytokines and accumulation of inflammatory cells that in the medium term have a more damaging effects on malignant rather than normal epithelium. Although such non-specific effect may contribute towards the efficacy of BCG, other mechanisms might be postulated to contribute to antineoplastic effect. Interestingly, BCG treatment has been reported to induce on tumour cells the expression of the tumour-associated MAGE antigens (Patard et al., 1995) and cross-reactive antigens, such as heat-shock proteins (Zlotta et al., 1997). To date the development of specific cytotoxic T cells to these antigens remains to be established.

In the light of existing knowledge on Th1-Th2 responses, it would be plausible that the success of BCG treatment in bladder cancer might be due to the preferential induction of Th1 responses. Indeed, cytokines classically associated with Th1 response, such as IL-2 and IFN-γ, have been detected in the urine of patients after intravesical administration of BCG, whereas IL-4 has proved difficult to detect. In addition the induction of interleukin-2 mRNA in peripheral-blood mononuclear cells of patients during therapy strongly correlated with clinical response (Kaempfer et al., 1996). Furthermore, in a mouse model of BCG therapy of bladder cancer treatment, success of the treatment appeared to be associated with a switch from a Th2 response to a Th1 response (McAveney et al., 1994).

Recently the results of a clinical trial of active specific immunotherapy using an autologous tumour cells-BCG vaccine reported in patients with colon cancer have been published (Vermorken et al., 1999). Two hundred and fifty-four patients with colon cancer were assigned randomly to postoperative active specific immunotherapy or no adjuvant treatment. Active specific immunotherapy was intradermal vaccination with irradiated autologous tumour cells with bacillus Calmette Guerin as an adjuvant. The 5.3 year median follow-up showed 44% risk reduction for recurrence in the recurrence-free period in all patients receiving active specific immunotherapy. The major clinical benefit was observed in patients with stage II disease, with a significantly longer recurrence-free period and 61% risk reduction for recurrences.

There is some evidence of a mechanistic relationship between the variable efficacy of BCG in prophylaxis against tuberculosis and its variable efficacy in tumour prevention and therapy (Grange et al., 1995). Several independent studies in different countries have shown that neonatal BCG vaccination affords some degree of protection against leukaemia and other childhood cancers, but only where it also protects against

tuberculosis. By contrast, among young adults to whom BCG afforded little protection against tuberculosis, it seemed to cause a slight increase in the frequency of cancer (Comstock, 1991), and it had a slightly negative effect when used as adjunct therapy for breast cancer (Early Breast Cancer Trialists' Collaborative Group, 1992).

Variation in the efficacy of BCG vaccination may be attributable to previous imprinting of the immune system by contact with environmental mycobacteria, leading to either a Th1 response, which is protective, or a mixed Th1/Th2 population of responsive T cells, which appears not to be effective and results in a phenomenon of indiscriminate tissue necrosis (Bretscher, 1992). It has been postulated that using other mycobacterial preparations it might be possible to suppress the indiscriminate necrosis and enhance Th1-regulated selective destruction of tumour cells (Grange et al., 1995).

1.5.3 IMMUNITY TO BACTERIAL DNA: CpG MOTIFS

Vertebrates have evolved innate immune defence mechanisms that recognise and respond to structural patterns that are specific to microbial molecules. Recently an increasing body of literature has examined one such pattern recognition system based on unmethylated CpG dinucleotides (Wagner, 1999).

Unmethylated CpG dinucleotides are common in bacterial DNA, but are under-represented and methylated in vertebrate DNA. Thus, this difference in the context of unmethylated CpG dinucleotides between vertebrate and microbial DNA provides a structural characteristic through which vertebrate leukocytes may detect and respond to infection.

In particular, it has been observed that optimal immune activation by microbial DNA is a consensus DNA motif in which an unmethylated CpG dinucleotide is flanked by two 5'purines and two 3'pyrimidines (CpG motifs) (Krieg et al., 1995).

1.5.3.1 CpG DNA effects on immune system

Several molecular, cellular and *in vivo* studies have been performed to evaluate the specific effects of CpG DNA on the cells of the immune system (Fig. 1.16). These studies have been performed using synthetic oligonucleotides containing CpG motifs or using bacterial DNA.

From these studies it appeared that DNA containing CpG motifs triggers humoral immunity by inducing B cell activation and IL-6 and IgM secretion. Furthermore, the strong activation effect of CpG oligonucleotides toward B cells also affected B cell apoptosis. A mechanism to maintain B cell tolerance is based on elimination of self-reactive B cells by apoptosis in response to surface IgM (sIg) cross-linking. Activation of mature B cells by antigens may thus require a signal that inhibits sIg-mediated

apoptosis. Signals to abrogate sIg-mediated apoptosis have been demonstrated to be generated by an appropriate costimulation, such as CD40 ligation, or stimulation with mitogens, such as LPS (Tsubata et al., 1993). Similar to CD40L and LPS, CpG oligonucleotides protected cells of the WEHI-231 B cell line from anti-IgM-mediated apoptosis (Yi et al., 1996a). This protection appeared to be associated with a reversion in anti-IgM-induced down-regulation of c-myc expression and up-regulation of myn, bcl-2 and bcl-x_L mRNA expression .

Apart from mitogenic effects on B cells, CpG DNA specifically activates antigen-presenting cells, the sentinels bridging innate and adaptive immunity (Bendelac et al., 1997). Indeed, CpG DNA directly activates monocytes and macrophages to express a full complement of costimulatory molecules, including CD40, CD80 and CD86 and to secrete cytokines, especially IL-12 and TNF-α at a level similar to those provoked by LPS (Ballas et al., 1996; Cowdery et al., 1996; Hallas et al., 1999; Stacey et al., 1996). It has been demonstrated that the CpG-driven secretion of high level TNF-α may cause harmful side-effects by promoting toxic shock, while the CpG-driven induction of IL-12 appear to be beneficial by promoting Th-1 polarised T cell responses (Lipford et al., 1997; Lipford et al., 1997). Interestingly, studies of sequence modifications of CpG oligonucleotides have permitted the creation of a CpG motif that effectively induced IL-12 but not TNF-α in macrophages, both *in vitro* and *in vivo* (Lipford et al., 1997; Lipford et al., 1997), avoiding TNF-α mediated harmful side effects.

In contrast to B cells and APCs, bacterial DNA and CpG oligonucleotides did not directly activate T cells, even if CpG oligonucleotides are able to costimulate antigen-reactive T cells that had received a stimulus. Indeed, it has been reported that, on cross-linking of T cell receptors (TCRs) via plastic-bound anti-CD3 mAb, CpG oligonucleotides costimulated T cells to produce IL-2, to express IL-2 receptors and to

proliferate and differentiate into CTLs (Chen et al., 1999). Interestingly, neither cytosine methylation, CG inversion to GC, nor flanking canonical CpG motifs with poly(G)stretches affected T cell costimulation, while all of these modifications abrogated direct activation of APCs. These data imply that T cell costimulation is mediated by signal pathways distinct from those characterising CpG oligonucleotides-mediated signaling via the chloroquine-sensitive endosomal pathway (see below).
However, an indirect activation of T cells by CpG DNA is mediated, as described above, by Th-1 type cytokines released by APCs. Indeed, the Th1 polarisation of T cells induced *in vivo* after treatment of mice with bacterial chromosomal DNA or CpG oligonucleotides was reported to promote resistance to infection with Listeria monocytogenes (Krieg et al., 1998; Elkins et al., 1999) or with Leishmania major in susceptible BALB/c mice (Zimmermann et al., 1998).

CpG oligonucleotides have been reported to rapidly induce lytic activity and IFN-γ secretion by NK cells *in vivo* (Shimada et al., 1986; Tokunaga et al., 1984; Cowdery et al., 1996). *In vitro* experiments demonstrated that the activation, CpG-mediated, of NK cells required the costimulation by cytokines, such as IL-12 and TNF-α, released from CpG -activated APCs (Hallas et al., 1999). Indeed, while CpG oligonucleotides alone failed to trigger IFN-γ production by NK cells, the presence of low concentrations of IL-12 seems to synergize with CpG DNA inducing secretion of high levels of IFN-γ (Chace et al., 1997).

In conclusion, these studies indicated that CpG DNA induces a predominantly Th1 pattern of immune activation. These potent and rapid immune-stimulating effects indicate that CpG DNA may be a "danger signal" able to activates innate immune defense (Krieg, 1996).

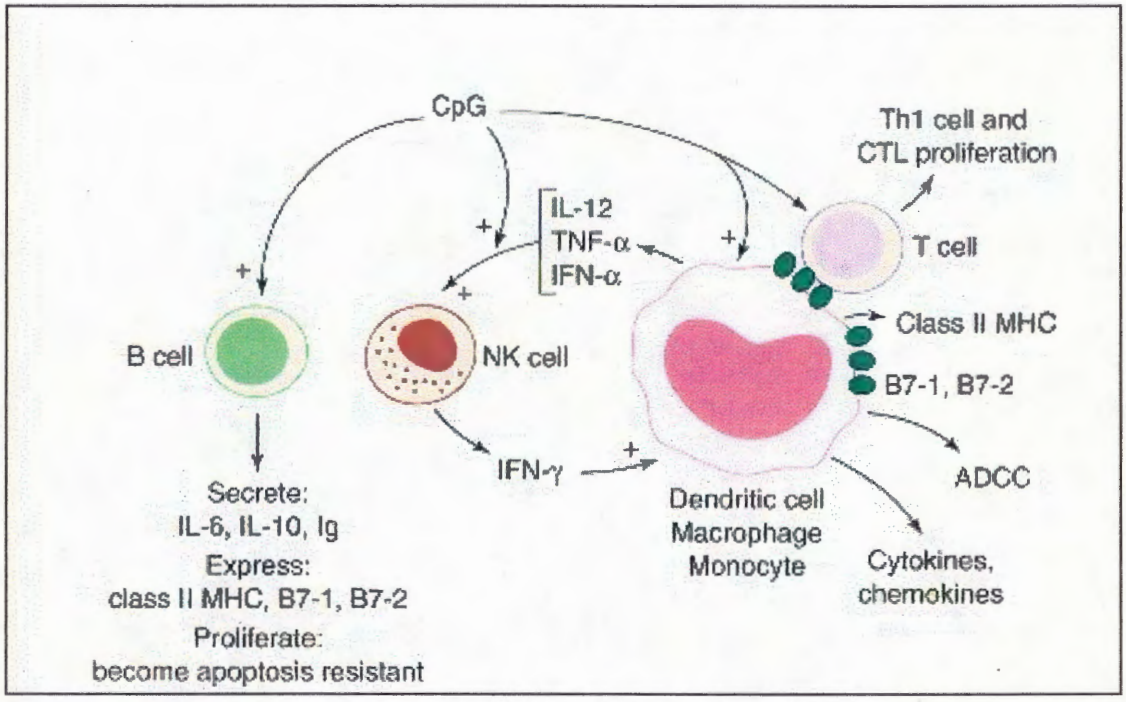


Fig. 1.16: CpG DNA effects on immune system

CpG DNA directly activates dendritic cells, monocytes and macrophages to express increased levels of costimulatory molecules and to increase antigen presentation and cross-priming. High levels of cytokines, especially Th1-like cytokines such as interleukin 12 (IL-12), interferon a (IFN-α) and tumour necrosis factor a (TNF-α), are expressed within a few hours, and monocytes and macrophages have increased antibody-dependent cellular cytotoxicity (ADCC) activity. NK cells are induced to express IFN-γ by these cytokines acting in concert with CpG and have increased lytic activity. B cells rapidly produce IL-6 and IL-10 and express increased level of costimulatory molecules. B cells rapidly enter the cell cycle and become resistant to some forms of activation-induced cell death. T cells are not directly activated by CpG, but because of the T helper 1 (Th1)-like cytokine environment and the increased antigen presenting cell (APC) activity, antigen-specific Th1 cells and cytotoxic T lymphocytes (CTL) are generated (from Krieg et al., 2000).

1.5.3.2 CpG DNA-mediated signaling

Even though it is not completely understood how CpG DNA sequences mediate cell-activation, some rate-limiting steps in signaling have been unraveled in APCs (Hacker et al., 1998) and in B cells (Yi et al., 1998b).

The rate-limiting step include cellular binding of CpG oligonucleotides, translocation into early endosomes, sensitivity to lysosomotropic compounds, and activation of the mitogen-activated protein kinase (MAPK) pathways, known to control transcriptional activities of AP-1 and of NFkB molecules.

Lysosomotropic compounds known to inhibit endosomal maturation, such as chloroquine, bafilomycin A, or monensin (Yoshimori et al., 1991; Fox, 1993), completely inhibited CpG DNA-driven antiapoptotic effects in WEHI-231 B cells (Macfarlane et al., 1998; Macfarlane et al., 1997) as well as CpG DNA-driven activation of APCs (Hacker et al., 1998). The lysosomotropic compounds did not affect cellular uptake of CpG oligonucleotides (Hacker et al., 1998), but completely destroyed CpG-driven downstream events, such as activation of stress kinases and of transcription factors such as AP-1 (Hacker et al., 1998).

Activation of APCs (Hacker et al., 1998) or B cells (Yi et al., 1998b) was mediated, at least in part, by rapid induction of mitogen-activated protein kinases of the "stress pathway" (Karin, 1995; Kyriakis et al., 1994) CpG-DNA triggered the kinase activity of JNK (Hibi et al., 1993) and of p38 (Han et al., 1994), a MAPK originally identified as a kinase activated by LPS. JNK activation and p38 activation were associated with subsequent phosphorylation of c-Jun and ATF2, components of the transcription factor AP-1 (Ray et al., 1989).

1.5.4 USE OF CpG DNA AS ADJUVANT FOR ANTITUMOUR RESPONSE

Conceptually, attempts to augment antitumour responses may benefit from the ability of bacterial DNA and CpG oligonucleotides to activate B cells, NK cells, macrophages and dendritic cells and the adjuvanticity of CpG oligonucleotides for CTL induction toward proteinaceous antigens. Furthermore, CpG oligonucleotides acted as adjuvant for Th1 polarisation of cellular responses.

In a study to identify the fraction of bacillus Calmette-Guérin (BCG) responsible for its antitumour activity, Tokunaga (Tokunaga et al., 1984) implicated the DNA component. Injection of a fraction, designated MY-1, purified from BCG containing 98% nucleic acid (70% DNA and 28% RNA) caused the IMC carcinoma of CDF1 mice and line 10 tumour of strain 2 guinea pigs to regress and prevented metastasis very effectively. MY-1 after digestion with RNAse was more effective than undigested MY-1 against tumours, while MY-1 digested with DNAse had reduced activity, suggesting that the DNA from BCG possessed strong antitumour activity under certain conditions.

Although studies using CpG oligonucleotides to augment antitumour responses are still in their infancy, initial reports appear promising.

Passive therapy with monoclonal antibodies (mAbs) can have antitumour effects in patients. NK cells and monocytes/macrophages participate in antibody-dependent cellular cytotoxicity (ADCC), which played a role in the response to antibody therapy. CpG oligonucleotides were assayed for enhancing antitumour effects of mAbs in a murine lymphoma model system. *In vivo*, there was a synergy between CpG oligonucleotides and the protective effects of antitumour mAbs. *In vitro*, CpG oligonucleotides increased ADCC (Wooldridge et al., 1997).

Using the secreted antibody from the 38C13 murine lymphoma to immunize against a protein tumour antigen, CpG oligonucleotides were assessed for their adjuvant activity

(Weiner et al., 1997). Adjuvancity of CpG oligonucleotides was as effective as that of complete Freund'adjuvant in promoting protection against tumour challenge.

Treatment with oligonucleotides containing a CpG immunostimulatory sequence was used recently to cure established neuroblastoma in syngeneic mice (Carpentier et al., 1999). Whereas none of the control animals survived the tumour challenge, 50% of mice treated with daily peritumoural injections of CpG oligonucleotides for 15 days were cured, and all of the animals treated with CpG oligonucleotides had reduced tumour growth. Intraperitoneally injection of CpG oligonucleotides was less efficient than peritumoural injection, suggesting that CpG oligonucleotides exert their effects locally and must reach a sufficient concentration at the tumour site.

1.6 AIM OF THE THESIS

The discovery of tumour antigens provided new opportunities for the development of therapeutic strategies against cancer. These approaches aim at increasing the physiologic recognition of tumour and at stimulating the effector components of the immune system which potentially contribute to the eradication of tumour cells. Most investigations in the field of cancer immuno-gene therapy have been related to the antitumour effect of cancer vaccines with transfected cytokine genes, major histocompatibility antigens and co-stimulatory molecules or to *in vivo* delivery of specific tumour antigens to antigen presenting cells.

The work presented in this thesis evaluated, as an approach to stimulating an immune response against tumours, the transduction of tumour cells with a bacterial gene, which represents a "danger signal".

Indeed, in less than a decade, the archetypal view that the immune system exists primarily to distinguish "self" from "non-self" has been replaced by the paradigm that the immune system functions primarily to distinguish dangerous from non-dangerous antigens. Presumably the immune system has evolved over millions of years to respond to structural patterns, that are specific to microbial molecules with a rapid activation of defenses best suited to fight microbial infection.

Mycobacterium tuberculosis is a major target in this fight and studies on complete Freund's adjuvant (CFA) indicate that the mycobacterium contains a number of substances that stimulate the immune response and promote Th1 differentiation.

In the light of the pivotal role of *M. tuberculosis* genes and their encoded proteins in linking innate and cell mediated adaptive immunity, these bacterial substances are promising candidates to be used as adjuvants for the development of effective therapeutic or prophylactic tumour vaccines. The immune response they elicit might

facilitate the activation of the immune system against tumour antigens and the eventual selective destruction of tumour cells through a specific immune response.

The *Ag38* gene of *Mycobacterium tuberculosis*, encoding a 38 kDa antigen, has been used in this work; on the basis of serologic and immunogenic studies this antigen has been defined to be one of the most potent immunogen among mycobacterial antigens. Indeed, this molecule, which is cell-wall associated and secreted from bacteria, may have a special role in protective immune mechanisms, since it is available for immune recognition at an early stage of mycobacterial infection, and may represent a strategy for the host to control the initial bacterial replication.

Using a retroviral vector, modified to express the leader and transmembrane sequences of the Nerve Growth Factor Receptor, the *Ag38* gene has been stably transduced into tumour cells and expressed as a protein bound to the cell surface.

The adjuvant effects of *Ag38* gene transduction in the response against poorly immunogenic tumours have been studied, using two different experimental models: one consisting of a tumour transplant model and one other consisting of a spontaneous tumour model.

In the first model, subcutaneously growing tumours and pulmonary metastases were induced in C57BL/6 mice by inoculation of murine melanoma cells. This tumour, as do many human tumours, expresses low levels of MHC class I antigens and different tissue-specific differentiation antigens, which, in melanoma patients, have been reported to represent potential targets for an immune response. This system, therefore, represents a suitable model for evaluating immunotherapeutic approaches to break tolerance to endogenous antigens.

In the second model, N202 transgenic mice carrying the proto-oncogene *HER2/neu* under the transcriptional control of the mouse mammary tumour virus promoter

(MMTV) have been used. The expression of proto-*neu* in the mammary tissue of these mice results in the development of spontaneous focal mammary tumours in all females. These tumours express the transformant oncoprotein, but are poorly immunogenic. This system was chosen as a realistic model to evaluate an immunotherapeutic strategy for cancer vaccination in individuals at risk for tumour development, with defined gene mutations.

The efficacy of the cancer vaccine, obtained by transduction of tumour cells with the *Ag38* gene, has been determined for its protection against the parental tumour.

The immunological responses induced by vaccination against tumour have been analysed and the involvement of both cellular and humoral immune effectors in tumour protection investigated in the two different models.

The recognition of endogenous tumour antigens stimulated by vaccination with transduced cells has been evaluated in particular in melanoma model, where a strong humoral response against specific antigens was observed.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MOLECULAR BIOLOGY

2.1.1 General procedures

All solutions employed for the preparation and manipulation of nucleic acids were made up using distilled water. All solutions were autoclaved before use or, in the case of thermolabile substances, filter-sterilised using a 0.22μm filter and stored in a sterile container. To reduce the chances of RNA degradation by contaminating RNAses, solutions used for RNA work were treated overnight with diethylpyrocarbonate (DEPC) as a 0.1% v /v solution and then autoclaved. Unless stated otherwise, all chemical reagents were supplied by Sigma (St. Louis, MO) or Merck (Darmstadt, Germany) and all enzymes used were purchased from New England BioLabs (Hitchin, Hertfordshire, UK) or Boehringer (Mannheim, Germany)

2.1.2 Concentration of nucleic acids

Nucleic acids were precipitated by adding 2.5 volumes of ethanol and chilled at -70°C for 30 minutes followed by centrifugation at 13,000 rpm for 15 minutes (Sepatech Biofuge, Heraeus Instruments). The pelleted nucleic acid was then washed with 70% v/v ethanol and then air dried before resuspension in distilled water or Tris-EDTA buffer (TE buffer: 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0).

2.1.3 Determination of nucleic acid concentrations

The absorbance of an aqueous solution of the nucleic acid was measured at 260 nm (Ultrospec 3000, Pharmacia Biotech). The convention used was that an absorbance of one unit is equivalent to a double stranded DNA concentration of 50 μg/ml and an RNA concentration of 40 μg/ml.

2.1.4 Preparation of competent *E. coli* for transformation

A single colony of JM101 *E.coli* was used to inoculate 2 ml of L-broth (1% w /v bacto-tryptone, 0.5% w /v yeast extract, 1% w /v NaCl) which was then incubated overnight at 37°C. From this 0.5 ml of the overnight culture was used to inoculate 50 ml of L-broth and this was incubated for several hours until an optical density of between 0.2 to 0.4 at 600 nm was achieved. The culture was cooled on ice for 10 minutes and the bacteria were then pelleted by centrifugation at 4000 rpm for 10 minutes at 4°C (Megafuge, Heraeus Instruments). The bacterial pellets were resuspended in 10 ml of 0.1M $CaCl_2$ and cooled on ice for 30 minutes. After recentrifugation, the bacteria were resuspended in 2 ml of 0.1M $CaCl_2$.

2.1.5 Transformation of bacteria

The plasmid DNA was added to 200 μl of competent *E. coli*. The suspension was cooled in ice for 45 minutes, warmed at 42°C for 2 minutes and then returned to ice for 2 minutes. 800 μl of L-broth was then added to the samples followed by incubation in a shaking incubator at 37°C for 1 hr to permit expression of the antibiotic resistance gene on the plasmid. The bacteria were then plated out onto 90mm petri dishes (Falcon) containing L-agar (L-broth with 1.5% w/v agar) with ampicillin (final concentration of 100 μg/ml). The plates were incubated overnight at 37°C.

2.1.6 Storage of bacteria

500 µl of a suspension of bacteria in the log phase of growth was diluted with 100 µl of 80% glycerol and stored in cryotubes (Nalgene) at -70°C. To resurrect bacteria from a frozen stock, a scraping was obtained using a sterile pipette tip and used to inoculate 10 ml of L-broth containing ampicillin. The bacteria were grown overnight in a shaking incubator at 37°C.

2.1.7 Small scale preparation of plasmid DNA ("miniprep")

Plasmid DNA was prepared from small cultures of bacteria using a Wizard Minipreps System (Promega, Madison, WI), following the protocol supplied by the manufacturer. This procedure was based on the alkaline lysis method for rapid extraction of plasmid DNA from bacterial cells (Birnboim et al., 1979) followed by the adsorption of DNA onto silica in the presence of high salt (Vogelstein et al., 1979).

Single bacterial colonies were inoculated into 10 ml of L-broth containing ampicillin and incubated overnight in a shaking incubator at 37°C. 1.5 ml of the overnight cultures were centrifuged at 13,000 rpm for 5 minutes and the bacteria were then resuspended in 200 µl of cell resuspension solution (50mM Tris-HCI pH 7.5, 10mM EDTA 100 µg/ml Rnase A). 200 µl of cell lysis solution (200mM NaOH, 1% SDS) was then added and mixed gently, followed by adding 200 µl of neutralisation solution (1.32M Potassium Acetate pH 4.8) which adjusts the sample to high salt binding conditions and causes precipitation of denatured proteins, SDS, cellular debris and chromosomal DNA. The samples were then centrifuged at 13,000 rpm for 10 minutes and the supernatants were then mixed to 1 ml with Wizard Minipreps DNA Purification Resin. The Resin/DNA mix was then transferred to a Wizard Minicolumn and vacuum suction was applied to cause flow through the minicolumn. After washing with 2 ml of column wash solution,

to remove salts, the DNA was eluted by applying 100 µl of distilled water to the Minicolumn.

2.1.8 Large scale preparation of plasmid DNA ("maxiprep")

Qiagen Plasmid Maxi kit (Qiagen, Hilden, Germany) was used which is based on the modified alkaline procedures (Birnboim et al., 1979) followed by binding of plasmid DNA to an anion-exchange resin. A single bacterial colony was used to inoculate a 2 ml volume of L-broth containing ampicillin which was incubated for 8 hr in a shaking incubator at 37°C. Then 1 ml of this culture was used to inoculate 500 ml of L-broth containing ampicillin which was then incubated overnight. The bacteria were pelleted by centrifugation at 6,000 rpm for 20 minutes (Sorvall, DuPont) and resuspended in 10 ml of resuspension buffer P1. 10 ml of lysis buffer P2 was then added and left at room temperature for 5 minutes. 10 ml of neutralisation buffer P3 (pre-chilled to 4°C) was added and the lysate incubated on ice for 20 minutes. The cell lysate was centrifuged for 30 minutes at 15,000 rpm and the supernatant was then filtered onto a QIAGEN-tip which had been pre-equilibrated with 10 ml buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% ethanol, 0.15% Triton X-100) and allowed to enter the anion-exchange resin by gravity flow. Under these conditions, the plasmid DNA binds to the anion-exchange resin. The resin was then washed with 60 ml of medium salt buffer QC (lM NaCl, 50mM MOPS, pH 7.0, 15% ethanol) to remove RNA, proteins and low molecular weight impurities. The DNA was eluted with 15 ml of high salt buffer QF (l.25M NaCl, 50mM Tris-HCl pH 8.5, 15% ethanol), and was then desalted by precipitation with 10.5 ml isopropanol. The DNA was pelleted by centrifugation at 9500 rpm for 30 minutes at 4°C , washed with 70% v/v ethanol, air dried and then dissolved in TE buffer.

2.1.9 Preparation of total RNA from cultured eukaryotic cells

RNA was obtained from adherent cell lines by employing the Rneasy Total RNA Purification Kit (Qiagen), which used a modification of the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski et al., 1987) and a silica gel-based membrane. Approximately $2x10^6$ cells were trypsinized, pelleted and then lysed in 350 µl of Lysis Buffer RLT (containing guanidinium thiocyanate and phenol). The lysate was centrifuged for 5 minutes at 13,000 rpm and the supernatant mixed vigorously with 1 vol 70% Ethanol. The mixture was then transferred to an Rneasy spin column and centrifuged at 10,000 rpm. After washing with Wash Buffer to remove salt, the RNA was eluted by applying 50 µl of DEPC-treated water to the Minicolumn.

2.1.10 Agarose gel electrophoresis of DNA

Gels were prepared by adding agarose (0.7 to 1.8% w /v) to 150 ml 1x TAE (Tris-acetate-EDTA) buffer (diluted from 50X TAE stock solution: 2M Tris base, 2M glacial acetic acid, 50 mM EDTA) and boiled in a microwave cooker for 5 minutes. On cooling to below 50°C, 2 µl of ethidium bromide stock solution (l0 mg/ml) was added. Gels were poured into a gel former with a well-comb in place. After setting, the gel was submerged in an electropheresis tank containing 1x TAE buffer. Loading buffer (1/6 volume of 6X stock solution: 0.25% bromophenol blue, 40% w/v sucrose in water) was added to the DNA solutions which were then transferred into the wells, and electrophoresis was performed at constant voltage (70 volts) for 2 to 4 h. The gel was transilluminated with short wave ultraviolet light and the DNA was visualised using an EagleEye II camera, monitor and printer (Stratagene). DNA fragments were sized by reference to a 1kb DNA Ladder (Gibco, Life Technology, Garthersburg, MD) which was run concurrently.

2.1.11 Purification of DNA restriction fragments

Agarose gels were visualised by UV transillumination and the bands of interest excised using a scalpel blade. The DNA was purified from the gel using the Wizard PCR Preps DNA Purification System (Promega), following the instructions provided by the manufacturer. The method is based on the binding of DNA to silica under high salt conditions (Vogelstein et al., 1979). The excised portion of the gel (300 mg) was dissolved in 1 ml Resin by heating at 65°C for 5 minutes. The Resin/DNA mix was then transferred onto a Minicolumn and after washing with 2 ml of 80% isopropanol, the DNA was eluted by applying 50 µl of water or TE buffer to the Minicolumn. 1 µl of the eluate was run on an agarose gel to confirm successful purification of the DNA fragment.

2.1.12 Digestion of DNA with restriction enzymes

Plasmid DNA was digested in volumes of 20 µl using 3 units of enzyme per µg of DNA for 60 minutes at 37°C. Appropriate buffers supplied by the manufacturer were used.

2.1.13 Removal of 5' terminal phosphate groups

To reduce re-ligation of the vector DNA in cases where cohesive ends were present, treatment with calf intestinal alkaline phosphatase (CIP) to remove the 5' phosphate groups of linear double stranded DNA was performed. At the end of a restriction enzyme digestion, 2 units of CIP were added to the reaction sample and incubated for a further 60 minutes at 37°C. The sample was then run on an agarose gel and the appropriate fragment was purified as described above.

2.1.14 Preparation of complementary DNA for analysis with PCR

First strand cDNA was generated from an RNA template using a GeneAmp RNA PCR Kit (Perkin Elmer, Norwalk, CT). An aqueous solution containing 1 μg of RNA in a volume of 20 μl was heated at 65°C for 10 minutes and then chilled on ice. The following were added to the RNA sample and incubated at 42°C for 15 minutes: 5mM $MgCl_2$, 1× PCR Buffer II, 1mM dGTP, 1mM dATP, 1mM dTTP, 1mM dCTP, 1U Rnase Inhibitor, 2.5U Reverse Transcriptase, 2.5μM oligo $d(T)_{16}$. For analysis with the polymerase chain reaction (rtPCR), 10 μl of the reaction mixture was used in each PCR sample.

2.1.15 Amplification of DNA sequences by the polymerase chain reaction

Polymerase chain reaction (PCR) was performed by cycling samples containing template DNA mixed with sequence-specific oligonucleotide primers through three temperature incubations in the presence of Thermus aquaticus (Taq) DNA polymerase (Perkin Elmer). These cycles were:

1. Denaturation of double stranded DNA
2. Annealing of primers to DNA
3. Extension of target sequences by Taq DNA polymerase.

The incubations were performed in a DNA Thermal Cycler (Perkin Elmer). The optimal cycle number and exact annealing and extension conditions were as described for each individual reaction (see Results). Primers were synthesised by PRIMM. Each reaction sample consisted of: template DNA (50 ng of plasmid DNA), 200μM dNTPs, 1x PCR buffer, 25 pmol of 5' primer, 25 pmol of 3' primer, 1U *Taq* DNA polymerase and distilled water added to a total volume of 50 μl. The reaction was then heated to 95°C for 5 minutes and then allowed to proceed through 30 to 35 cycles of denaturation, annealing and extension to produce the required degree of amplification. The amplified

PCR products were evaluated by mixing 10 µl of the reaction mixture with 2 µl of 6x loading buffer stock solution and were then run on an agarose gel.

2.1.16 Ligation of PCR products

For ligation of *Ag38* DNA into the pLTMSN vector, the ligation reaction was performed in a volume of 15 µl using 1 unit of T4 DNA ligase and ligase buffer (50mM Tris-HCl pH 7.8, 10mM $MgCl_2$ l0mM DTT, 1mM ATP, 25 µg/ml BSA). The molar ratio of vector to insert was in the range of 1:4 to 1:10. The reaction mixture was incubated overnight at 16°C and was then transformed into competent *E. coli* JM101.

For ligation of *TRP2* DNA into the PCR3.1 Uni vector, the TA cloning Kit (Invitrogen, San Diego, CA) was used. This system takes advantage of the nontemplate-dependent activity of Taq polymerase which adds a single deoxyadenosine to the 3' termini of the double stranded molecules. The linearised vectors which are supplied possess single overhanging deoxythymidine residues at the 3' termini, thus allowing the PCR product to ligate efficiently with the vector. The ligation reaction was perfomed according to the manufacturer's instructions in 10 µl volumes consisting of : lµl of l0x ligation buffer (60mM Tris-HCl pH 7.5, 60mM $MgCl_2$ l50mM NaCl, 1 mg/ml BSA, 70mM β-ME, 1mM ATP, 20mM DTT, 10mM spermidine), 1 µl T4 DNA ligase, 2 µl linearised vector (60 ng), 1 µl PCR reaction mixture and 5 µl distilled water. The reaction mixture was incubated overnight at 16°C and was then transformed into competent *E. coli* (TOP10F' strain) and plated onto L-agar containing ampicillin.

2.1.17 Agarose gel electrophoresis of RNA for "Northern" transfer

Separation of RNA was performed in formaldehyde denaturing gels. Agarose was dissolved in DEPC-treated water by boiling to 100°C. On cooling to 60°C, l0x MEN solution (200mM MOPS, 10mM EDTA, 50mM sodium acetate, pH 7.0) and

formaldehyde solution were added to give a final concentration of 1.4% w/v agarose, lx MEN and 2.2M formaldehyde. Gels were cast as described earlier and were submerged in lx MEN running buffer. RNA samples were prepared for electrophoresis by precipitation and resuspension in lx MEN buffer followed by addition of formaldehyde and formamide to final concentrations of 2.2M and 50% v /v, respectively, such that each sample had a total volume of 12.5 µl. 15 µg of RNA markers (Gibco) were also precipitated and treated as for the RNA samples. The RNA samples were heated to 70°C for 5 minutes and cooled immediately on ice. 0,5 µl of ethidium bromide stock solution and 1/10 volume RNA loading buffer (50% glycerol, 1mM EDTA, 0.25% w/v bromophenol blue, 0.25% xylene cyanol) was added before loading into the wells. Electrophoresis was performed at 20V until the dye front had travelled 3/4 of the length of the gel.

2.1.18 Capillary transfer of RNA from agarose gels

RNA fragments separated by agarose gel electrophoresis were transferred to Hybond-N+ membranes (Amersham, Buckinghamshire, UK) prior to hybridisation with radiolabelled DNA probes (Alwine et al., 1977). Following visualisation of the RNA under UV transillumination the gel was placed in denaturing solution of 0.05M NaOH for 30 minutes and then soaked in 20x SSC (saline-sodium citrate) solution (3M NaCl, 0.3M sodium citrate, pH adjusted to 7.0 with NaOH) for 30 minutes. The gel was then placed on a raised platform covered with two sheets of Whatman 3MM filter paper pre-soaked in 20x SSC were the ends of the filter paper extending below the platform into a reservoir of 20x SSC. A sheet of Hybond-N+ membrane was then placed in direct contact with the gel. Five sheets of filter paper, pre-soaked in 20x SSC, was then placed on top of the membrane followed by a box of paper towels compressed with a 1 kg weight. The assembly was left overnight to allow transfer of RNA. Completion of

transfer of RNA from the gel was checked by UV transillumination of the filter. The filter was then exposed to UV irradiation (UV Stratalinker 1800, Stratagene) to enable cross-linking of the RNA onto the filter.

2.1.19 Labelling of DNA probes with radio-isotopes

Double-stranded fragments for probing RNA blots were labelled using mixed hexadeoxyribonucleotide primers of random sequence (Feinberg et al., 1983), employing reagents supplied in a kit (Boehringer). An aqueous solution containing 50 ng of DNA was boiled at 100°C for 3 minutes and then mixed in a buffered solution containing dATP, dGTP, dTTP and random hexadeoxyribonucleotides in a total volume of 50 µl. Labelling was performed at 37°C for 1 hr with 1.85 MBq of [α-32p]dCTP using 10 units of Klenow subunit of DNA polymerase. The labelling mix was then passed down a G-50 Sephadex column to remove unincorporated nucleotides. The labelled product was then boiled for 5 minutes with 200 µl of salmon sperm DNA (10 mg/ml) before being added to the hybridisation solution.

2.1.20 Hybridisation of RNA immobilised on filters

Blotted filters were wetted in 2x SSC and then transferred to rotating glass bottles. 20 ml of prehybridisation buffer was then added to the bottle and incubated at 42°C in a rotating Techne oven for at least 6 h. Denatured radiolabelled probe was then added and incubated overnight at 42°C. Following hybridisation, the blots were washed once in 5x SSC for 15 minutes and then twice in 0.1x SSC/0.1% w/v SDS for 15 minutes each. After the final wash the filters were wrapped in plastic sheets and exposed to Kodak X-Omat films with intensifying screens at -70°C.

2.1.21 Automated sequencing of DNA by the chain termination method

Sequencing reactions were performed using an ABIPRISM Dye Terminator Cycle Sequencing Reaction kit (Perkin Elmer), according to the protocol provided by the manufacturers which is outlined below. Enzymatic extension reaction was performed in an asymmetric PCR using a single primer, dye-labeled terminators and the AmpliTaq DNA polymerase FS, a mutant form of Taq DNA polymerase which has essentially no 5'→ 3' nuclease activity and a drastically reduced discrimination for dideoxynucleotides.

Each reaction sample consisted of: template DNA (0.5 μg of DNA plasmid), 3.2 pmol Primer, 6 μl Terminator Ready Reaction Mix, which contains AmpliTaq DNA Polymerase FS, A-Dye, C-Dye, G-Dye and T-Dye labeled terminators, dITP, dATP, dCTP, dTTP, Tris-HCl pH 9, $MgCl_2$, thermal stable pyrophosphatase and H_20 to 20 μl volume.

Extension reaction was performed in a DNA Thermal Cycler with a denaturation step at 96°C for 4 min and 25 cycles at 96°C for 30 sec, 50°C for 20 sec, 60°C for 4 min followed by a single cycle at 60°C for 3 min. After precipitation of DNA, samples were loaded onto a polyacryilamide gel and the samples were analyzed on an ABI PRISM 377 DNA Sequencer (Perkin Elmer) which was operated by the Sequencing Service of Istituto Nazionale Tumori, Milan.

2.1.22 *In vitro* coupled transcription-translation

In vitro transcription-translation was performed in a single step using a TnT T7-coupled reticulocyte lysate system (Promega), according to the protocol provided by the manufacturers which is outlined below.

The DNA template (1 μg DNA plasmid) was incubated for 120 minutes at 30°C in a 50 μl reaction mixture containing 25 μl rabbit reticulocyte lysate, 1 μl T7 TnT RNA polymerase, 1 μl amino acids mixture 1mM minus methionine, 40 U Rnasin, 2 μl TnT reaction buffer and 4 μl translation-grade ^{35}S-methionine (1000 Ci/mmol; 10 mCi/ml; Amersham). A Sephadex G-25 column (Amersham) was used to purify the translated product and the percentage incorporation of ^{35}S-methionine was determined by TCA precipitation. The reaction was stored at -20°C until needed.

2.2 CELL BIOLOGY

2.2.1 Eukaryotic cell culture - General procedures

All manipulations involving cell culture were carried out in a sterile environment provided by a laminar flow hood. All tissue culture reagents were filter sterilised by passage through a 0.22μm filter and stored in sterile autoclaved containers. All cell culture media were purchased from Sigma.

The cell lines used in this work are listed in Table 2.1. Adherent cell lines were grown as monolayers in plastic tissue culture flasks or dishes (Falcon) in DMEM supplemented with 10% v/v fetal calf serum (Gibco) (previously heat-inactivated by incubating at 56°C for 30 minutes) and incubated at 37°C in 8% CO2. Cells were grown until just subconfluent (approximately 2 to 4 days) and were subcultured 1:10, using trypsin (0.05% w/v)/5mM EDTA to detach the cells. Cells grown in suspension were cultured in RPMI+l0%FCS and were subcultured 1:10 every 3 days. All cell lines were tested routinely for Mycoplasma infection (performed by technical personnel) and only cell lines in which infection was undetectable were used for experiments.

Cell counts were performed using a Burker's chamber and an inverted microscope.

2.2.2 Storage and recovery of cells stored in liquid nitrogen

Cells were trypsinised, pelleted and resuspended at approximately 10^7 cells/ml in fetal calf serum containing 10% v /v dimethylsulphoxide (DMSO), the presence of which prevented the formation of damaging ice crystals. 1 ml aliquots were transferred to 1.5 ml Nalgene cryotubes which were then placed in a -70°C freezer overnight. Frozen cells were then transferred to liquid nitrogen tanks (-196°C) the following day.

Recovery of cells from liquid nitrogen storage was performed by rapid thawing in a 37°C water bath. Thawed cells were washed in 10 ml of medium, harvested by centrifugation (1200 rpm for 5 minutes) and were then transferred to 25cm^2 flasks containing fresh culture medium.

2.3 GENE TRANSFER INTO EUKARYOTIC CELLS

2.3.1 Growth selection in Geneticin (G418 sulphate)

Geneticin is an aminoglycoside antibiotic related to Gentamicin and is toxic to both prokaryotic and eukaryotic cells. Introduction of the neomycin phosphotransferase gene into eukaryotic cells can confer resistance to Geneticin added to normal medium. Geneticin (Gibco) was added to DMEM to a concentration of 1 mg/ml for selective growth of B16 and B16.B78 cells, 0.5 mg/ml for N202.1A cells and 0.8 mg/ml for CHO-K1 and NIH-3T3 cells, these being the concentrations previously determined to be optimal for selective inhibition of growth of these cells.

2.3.2 Calcium phosphate/DNA co-precipitation

This method involves mixing DNA with $CaCl_2$ and a phosphate buffer to form a fine precipitate which is deposited onto the cultured cells (Graham et al., 1973). Twenty-four

hours prior to transfection, $5x10^5$ cells were plated out in a $25cm^2$ flask. 10 μg of the plasmid DNA to be transfected were made up to 300 μl using sterile distilled water followed by the addition of 100 μl of 2.5M $CaCl_2$ and 600 μl of sterile distilled water. 1 ml HEPES-PO_4 was then added dropwise to the mixture, during which time a fine precipitate became visible. The sample was incubated at room temperature for 30 minutes and then added dropwise to the medium in the cell culture flask. On the following day the medium was removed and replaced with fresh medium.

2.3.3 Liposome/DNA complexes

Lipofectin reagent (Gibco) was used to form lipid-DNA complexes which were able to fuse with cells resulting in efficient uptake of the exogenous DNA (Felgner et al., 1987). Lipofectin is a 1:1(w/w) formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphotidylethanolamine (DOPE) in water. Twenty-four hours prior to transfection, approximately $5x10^5$ cells were plated out in a $25cm^2$ flask to produce a culture which was approximately 40-60% confluent. 5 μg of the plasmid DNA to be transfected were made up to 100 μl in serum-free DMEM. In a separate tube, 20 μl of Lipofectin was combined with 80 μl of serum-free DMEM. The DNA solution and the diluted Lipofectin were then combined and incubated at room temperature for 15 minutes. During this time, the growth medium was removed from the cell culture flask and the cells washed twice with 5 ml of serum-free DMEM. After incubation, the DNA/Lipofectin mixture was mixed with 1.8 ml of serum-free medium and then added to the cells and incubated at 37°C for 24h. The DNA-containing medium was then replaced with fresh normal growth medium. In order to obtain stable transfectants, the cells were split into selection medium after another 48 hr .

2.3.4 Retroviral-mediated gene transfer

2.3.4 (1) Generation of producer cell lines

Since to make stable lines it is important to infect rather than transfect the packaging cells (Miller et al., 1989), producer cell lines have been generated by infection with the supernatant obtained after transient transfection of a different packaging line.

Amphotropic packaging cells, GP+envAM12 (Markowitz et al., 1988b), were transfected with 10 μg of vector plasmid DNA using the calcium phosphate/DNA co-precipitation method. 48 hr after transfection the supernatant from AM12 cells was used to infect the ecotropic packaging cells GP+E86 (Markowitz et al., 1988a) in the presence of polybrene (at a final concentration of 8 μg/ml) to enhance virus-cell surface interaction. The cells were split into selection medium 72 hr after infection. After 14 days, individual colonies and the bulk culture which survived selection were recovered and expanded.

2.3.4 (2) Harvest of retroviral stocks

Virus was harvested from producer cells by exposing 8 ml of fresh medium to $5x10^6$ cells in a $75cm^2$ flask and harvesting the medium 16 hr later. The medium was filtered (0.45μm) to remove cell debris and was either used for infection of target cells immediately or was stored frozen at -70°C.

2.3.4 (3) Infection of target cells by retroviral stocks

Tumour cells to be infected ("target cells") were plated out in a $25cm^2$ flask to produce a culture which was approximately 40-60% confluent the following day. The medium was removed and replaced with 2 ml of viral supernatant which contained polybrene (at a final concentration of 8 μg/ml) to enhance virus-cell surface interaction. After 2 hr at

37°C, 5 ml of media was added and cells incubated 16 hr at 37°C. The viral supernatant was then removed and replaced with fresh normal growth medium. The cells were grown for another 48 hr and then split into selection medium. Resistant surviving colonies were visible after about 14 days and these were lifted using trypsin- transferred to individual wells of a 24-well plate, followed by expansion into larger cell culture flasks.

2.3.4 (4) Determination of viral titre

Supernatant from individual G418-resistant colonies and from the bulk culture of producer cells was tested for the viral titre as the capacity to provide G418 resistance to NIH3T3 fibroblasts. Fibroblasts were plated out in a $25cm^2$ flask to produce a culture which was approximately 40-60% confluent the following day. The medium was removed and replaced with 2 ml of viral stocks dilutions (10^{-3}, 10^{-4}, 10^{-5}) in the presence of polybrene, as above. After 2 hr at 37°C, 5 ml of media was added and 24 hr later the cells were transferred in media containing 800 μg/ml G418. G418-resistant clones were counted 14-18 days later.

2.4 ASSAYS

2.4.1 Flow cytometry

Flow cytometry was used to detect the expression of cell surface or intra-cellular molecules on tumour cells. Adherent tumour cells were trypsinised and washed twice in ice-cold PBS and then resuspended in PBS-0.03% BSA at a concentration of $5x10^6$ cells/ml. For detection of cell surface molecules 100 μl of the cell suspension was incubated at 4°C for 45 minutes with the primary monoclonal antibody (at the optimal

pre-determined concentration) or with polyclonal antiserum, as specified in Results. As a negative control, PBS-0.03% BSA was added in place of the primary antibody. After washing and spinning down (1200 rpm for 5 minutes) the cells twice with PBS, cells were resuspended in PBS-0.03% BSA and an appropriate FITC-conjugated secondary antibody (used at dilutions indicated in Table 2.3) were added to the cells and incubated at 4°C for 30 minutes. After two washes with PBS, the cell pellet was resuspended in 300 µl of PBS and the cells were then analysed using a FACScan or a FACScaliber flow cytometers (Becton Dickinson, Mountain View, CA). For intracellular staining, permeabilisation was obtained by treatment with 50% methanol solution for 30 min at 4°C or with 3% paraformaldehyde (10 min at room temperature) and PBS-Digitonin (50 µg/ml) (10 min at room temperature).

Multiparameter analysis was used to detect expression of cell surface markers and cytokine production by murine splenocytes. Splenocytes were cultured for 21 hr with immobilised anti-CD3 mAb (10 µg/ml) and 1 µM monensin added for the last 12 hr (Sigma). Cells were washed twice in PBS and then fixed with 4 % paraformaldehyde for 5 min at room temperature and permeabilised with PBS-0.2% saponin for 15 min at room temperature. Cells were triple-stained with Cy-Chrome-labeled, FITC-labelled and PE-labelled monoclonal antibodies. Isotype-matched FITC-, PE- and Cy-Chrome-conjugated mAbs were used for background determination. All mAbs were purchased from Pharmingen (San Diego, CA) and diluted as indicated in the supplier's sheet.

2.4.2 Detection of cytokines using the enzyme-linked immunosorbent assay (ELISA)

ELISA was performed using optimised ELISA kit purchased from Genzyme (Cambridge, MA), Euroclone (Paignton, Devon, UK) and Pharmingen, using the protocol and reagent's dilutions recommended by the manufacturers as outlined below.

Kits purchased from Genzyme or Euroclone supplied also plates pre-coated with the appropriate antibody, ready to use. When working with the kit from Pharmingen, the coating was performed by adding 100 µl aliquots of purified antibody diluted in coating buffer to wells of a protein binding 96-well plate (Nunc Maxisorp) and by incubation overnight at 4°C. In all experiments plates were then washed twice by immersion in PBS/Tween (0.05% v /v Tween-20 in PBS) and each well was then filled with 200 µl PBS/10%FCS and left at room temperature for 2 h. After two washes with PBS/Tween, l00 µl of each test sample were placed in duplicate in individual wells and incubated at room temperature for 2 hr. For standards, a range of diluted recombinant cytokine was used. The plates were then washed four times in PBS/Tween followed by addition of 100 µl of biotinylated antibody, diluted in PBS/10%FCS, to each well. After incubation at room temperature for 1 hr, the plate was washed six times with PBS/Tween. Each well was then filled with 100 µl of HRP-conjugated streptavidin. After incubation at room temperature for 30 minutes the plate was washed eight times with PBS/Tween followed by addition of 100 µl of the TMB substrate (Sigma) to each well. The plates were read at OD 450 nm using a Microplate reader 550 (Biorad).

2.4.3 Assay for cytotoxic lymphocyte activity (CTL assay)

This assay of cytotoxicity was performed 5 days after *in vitro* or *in vivo* restimulation with irradiated tumour cells.

2.4.3 (1) Preparation of splenocytes

Fresh splenocytes were harvested from mice and spleens removed using aseptic technique. The spleens were gently teased apart in 10 ml of RPMI in a petri dish to obtain a cell suspension. The cells were pelleted by centrifugation (1500 rpm for 5 min) and then resuspended in 3 ml aqueous ammonium chloride 0.17M for 10 min to lyse red blood cells. The suspension was then restored to isotonicity by adding 20 ml of RPMI. After centrifugation (1500 rpm for 5 min), the cells were suspended in RPMI.

2.4.3 (2) Restimulation in vitro

Splenocytes were resuspended to a concentration of 5×10^6 cells/ml in RPMI medium containing 2-β-mercaptoethanol (final concentration of 5×10^{-5}M) and recombinant IL-2 (final concentration of 25 units/ml). 1 ml of the cell suspension was then placed in each well of a 24-well plate.

Adherent tumour cells were trypsinised, washed in growth medium and resuspended in 10 ml of PBS. The suspension of cells in PBS was then irradiated (20,000 rad) and, after centrifugation, resuspended in RPMI at a concentration of 5×10^4 cells/ml. 1 ml aliquots were added to each of the wells in the 24-well plate and the cell mixture was incubated at 37°C for 5 days.

2.4.3 (3) Labelling and lysis of target cells

Approximately 2×10^6 adherent cells were trypsinised, washed in medium and resuspended in 300 µl of medium. 100 µl of sodium [^{51}Cr]-chromate (3.4 MBq) was added to the cells and incubated for 45 minutes at 37 °C, and then the cells were washed three times in 10 ml of serum-free medium followed by resuspension in RPMI at a

concentration of 5×10^4 cells/ml. 100 µl of the labelled-cell suspension was then added to each well of a round-bottomed 96-well plate.

Splenocytes were centrifuged (1500 rpm for 5 min) and resuspended at concentrations of 5×10^6, 2.5×10^6 and 1.25×10^6 cells/ml and 100 µl of each of these dilutions were added to the target cells to give effector:target ratios of 100, 50 and 25:1. The samples for each effector:target ratio were tested in replicates of three to four. Also included in the assay were samples to which no lymphocytes had been added, to indicate the level of spontaneous lysis. In addition, samples to which 100 µl of 2% Triton were added were used to indicate maximum lysis. After incubation at 37 °C for 4 hr, the plate was centrifuged at 1500 rpm for 5 min. 100 µl of supernatant was then carefully aspirated from each well and the radioactivity determined using a beta counter (LS1801 Beckman). The percentage of lysis was determined as:

% Lysis = (T-S) / (M-S) × 100, where

T is the radioactivity (cpm) released into the supernatant in the presence of effector cells,

S represents spontaneous release in the absence of effector cells,

M represents maximal lysis.

2.4.4 Assay for complement-dependent cytotoxicity

This assay was used to determine the antibody-mediated cytotoxicity in sera from vaccinated mice. The target cells were labelled with [^{51}Cr] as in the CTL assay and 2×10^3/well were incubated with mice serum at serial dilutions for 45 min at 37°C. After washing, rabbit complement (Cedarlane, Hornby, Ontario, Canada) was added, and after 2 hr incubations at 37°C supernatants were harvested for determination of released radioactivity. As above, I also included in the assay samples to which no serum had

been added, to indicate the level of spontaneous lysis, and samples to which 2% Triton was added, to indicate maximum lysis. Percent cytotoxicity was calculated as follows:

% Lysis = (Ab-S) / (M-S) × 100, where

Ab is the radioactivity (cpm) released into the supernatant in the presence of serum,

S represents spontaneous release in the absence of serum,

M represents maximal lysis.

2.4.5 Lymphocyte proliferation assay

The proliferation assay was used to determine the ability of splenocytes to proliferate in the presence of tumour cells. Splenocytes were resuspended to a concentration of 1×10^6 cells/ml in RPMI medium containing 2-β-mercaptoethanol (final concentration of 5×10^{-5}M), recombinant IL-2 (final concentration of 25 units/ml) and with or without irradiated (3000 rads) fresh syngeneic splenocytes at concentration of 2.5×10^5 cells/well, as feeder cells. Irradiated (20,000 rad) tumour cells were resuspended in RPMI at a concentration of 1×10^5 cells/ml and 100 µl aliquots were added to each of the wells in the 96-well plate as stimulator cells. After 3 days of co-incubation at 37°C, cultures were pulsed with 1 µCi [^{3}H]thymidine (Amersham) incubated at 37°C. After 8 hr, cells were harvested and transferred to a filter (Spot-on filtermat; Pharmacia Biotech). Tritiated thymidine uptake was determined by a 1205 betaplate liquid scintillation counter (Wallace Inc.). Counts per minute were expressed as means of triplicate cultures.

2.4.6 Tumour cell-proliferation assays

In vitro inhibition of tumour cell growth was determined using an SRB (sulphorhodamine B) assay. SRB stains for cellular proteins. Cells were seeded at 1.5×10^3 cells/well in 96-well microplates in 100 µl of culture medium alone. After 24 hr

incubation at 37°C, the specific treatment diluted in 100 µl of final volume was added in each well and cells were incubated as indicated in the Results. Recombinant IFN-γ or IL-4, added as treatments in some experiments, were purchased from Peprotech (London, UK). After removing culture medium, cells were fixed in 50% trichloroacetic acid at 4°C for 1 hr, washed five times with distilled water and stained with 1% acetic acid-0.4 % (w/v) SRB solution at room temperature; after 30 min, plates were washed five times with 1% acetic acid and air-dried. SRB bound to cellular proteins was dissolved by addition of 10 mM Tris-HCl, pH 10.5, to each well. Absorbance at 550 nm, proportional to the number of cells attached to the culture plate, was measured by spectrophotometry. Each test was performed in quadruplicate.

2.5 BIOCHEMICAL STUDIES

2.5.1 Total cellular protein extraction

Confluent cultures cells were trypsinised, washed twice in ice-cold PBS and solubilised by incubation at 0°C for 1 hr in solubilisation buffer, containing 25 mM Tris-HCl pH 7.4, NP40 1%, and protease inhibitors (10 µg/ml Aprotinin, 5 µg/ml leupeptin and 1 mM PMSF). The lysate was then cleared by centrifugation at 13000 rpm for 5 minutes. The samples were stored at -20°C until needed.

2.5.2 Cell membrane protein extraction

Membrane protein extract was obtained according to the technique of Landowski et al. (Landowski et al., 1995) with minor modifications: 1.5×10^8 cells were lysed in ice-cold 25mM Tris/0.3M sucrose (pH 7.4) buffer, including protease inhibitor, by sonication with 5×5-sec bursts on ice, followed by centrifugation at 1000 rpm for 5 min at 4°C.

This step was repeated 3 times and the supernatant was collected. After centrifugation at 47,000 rpm for 90 min at 4°C (Ultracentrifuge L8-70, Beckman), the pellet was resuspended and rotated end-over-end for 12-16 hr at 4°C in 50mM Tris/150mM NaCl, pH 7.4, buffer containing 1% NP40 detergent and protease inhibitor. Insoluble material was pelleted by centrifugation at 47,000 rpm for 60 min and the supernatant, corresponding to the cytosolic fraction, was discarded. To obtain the membrane fraction, the pellet was resuspended and rotated end-over-end for 1 hr at 4°C in 50mM Tris/150mM NaCl pH7.4 buffer containing 1% β-octyl detergent and protease inhibitor. Insoluble material was pelleted by centrifugation at 13,000 rpm for 5 min and the supernatant was recovered and stored at -20°C.

2.5.3 Protein determination

Protein concentrations were determined by the bicinchoninic acid (BCA) test developed by Pierce (Rockford, IL). Protein content was extrapolated from a standard curve with bovine serum albumin standard solution (1mg/ml).

2.5.4 Cell surface radiolabelling

Surface radiolabelling was obtained by membrane lactoperoxidase-catalysed iodination. Confluent cultures of cells were trypsinised, washed twice, placed in Dulbecco's PBS for 3 hr before labelling. Cells were then incubated for 10 min at room temperature in a total volume of 2 ml PBS containing 20 µl ^{125}I (2mCi), 195 µg lactoperoxidase and 25 µl of 0.03% H_2O_2. After 5 min another 25 µl of H_2O_2 were added. The reaction was stopped by adding 10 ml of cold PBS and the cells were then washed 3 times with the same buffer. After extraction of the total cellular proteins, the percentage of labelling was determined after TCA precipitation using a gamma-counter (Cristal II Multidetector Ria System, Packard).

2.5.5 Immunoprecipitation

The protein extracts were precleared by adding Gamma Bind Plus Sepharose (Pharmacia Biotech, Uppsala, Sweden) to the cell extract which then was rotated end-over-end for 1 hr at 4°C. The precleared extract was recovered by centrifugation and aliquots of ~1×10^7 cpm of labelled proteins or 4 mg of unlabelled lysate were incubated with sera or antibodies and rotated end-over-end at 4°C. After 2 hr, 50 µl of Gamma Bind Plus Sepharose was added and samples were incubated for another 2 hr under the same conditions. The Sepharose-conjugates were washed 3 times with solubilisation buffer and precipitated molecules were extracted in Laemmli SDS reducing sample buffer (0.05M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue) by heating for 10 min at 95°C and centrifugation.

Immunodepletion experiments were performed by sequentially treating solubilised extracts with antibodies as described in Results, using the supernatant from each reaction for each subsequent immunoprecipitation. After 3 cycles of such absorptions, proteins remaining in the supernatants were subjected to a final antibody immunoprecipitation.

2.5.6 Electrophoretic procedures

Protein samples were analysed by Laemmli SDS gel electrophoresis system (Laemmli, 1970) using homogenous (15% or 10% or 8% acrylamide) gels or exponential gradient (5-15% acrylamide) gels. Gels containing the desired concentration of acrylamide were prepared by diluting acrylamide in a buffer containing: 0.375M Tris pH8.8, 0.1%SDS, 0.1%Ammonium persulfate and 0.1%TEMED in the Resolving gels and 0.125M Tris pH6.8, 0.1%SDS, 0.1%Ammonium persulfate and 0.1%TEMED in the Stacking gels. After denaturation of the proteins by heating at 95°C for 5 min in Laemmli SDS reducing sample buffer, samples were loaded into the wells of vertical gels and run in

electrophoresis buffer (25mM Tris, 250mM glycine pH 8.3 and 0.1% SDS) at a constant current of 25 mA. Unlabelled (Biorad) or methylated ^{14}C-labelled (Amersham) standard proteins were loaded and run concurrently in the same gel to estimate, by comparison with their mobilities, the molecular weights of the protein samples. After separation, the samples were analysed by autoradiography or Western blot.

2.5.7 Autoradiography and Western blot

When labelled proteins were resolved the gels were fixed in 10% trichloroacetic acid, dried and autoradiographed at -70°C with intensifying screens.

Unlabelled proteins were transferred from the gels to nitrocellulose membranes by Western blot. After equilibration in transfer buffer (39mM glycine, 48mM Tris base, 0.037% SDS and 20% methanol), the gels were sandwiched between a sheet of nitrocellulose membrane (Hybond-C super, Amersham) and several sheets of blotting paper, then assembled into a blotting apparatus and electroeluted in transfer buffer, applying a current of 0.65 mA/sq. cm of gel for 2 hr. The blots were then blocked by gently shaking in PBS-5% non fat dry milk overnight at 4°C and proteins detected by incubation of the blots with primary antibody or anti-serum diluted at optimal pre-determined concentration (see Table 2.2) in PBS-5% non fat dry milk for 2 hr at room temperature. After 3 washing with PBS-0.1% Tween, the blots were incubated with the appropriate peroxidase-coupled secondary antibody diluted 1:3000 in PBS-0.1%Tween-5% non fat dry milk for 1 hr at room temperature. After washing proteins were visualised using the ECL detection system (Amersham).

2.5.8 Protein sequencing

For protein sequencing, samples obtained from immunoprecipitation were electrophoresed onto 10% acriylamide and then transferred to polyvinylidene difluoride

(PVDF) membranes, strong solid phase supports which bind proteins hydrophobically and are inert to most solvents (Matsudaira, 1987). The gels were soaked in transfer buffer (10mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11.0) for 5 min to reduce the amount of Tris and glycine. During this time a PVDF membrane was rinsed with 100% methanol and stored in transfer buffer. The gel, sandwiched between a sheet of PVDF membrane and several sheets of blotting paper, was then assembled into a blotting apparatus and electroeluted in transfer buffer. The PVDF membrane was washed in deionised H_2O for 5 min, stained with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min and the band was cut out with a clean razor. Sequencing of the protein corresponding to the eluted band was operated using an Applied Biosystems model 470 sequenator by the Institute of Veterinary and Biochemical Physiology, University of Milan.

2.6 ANIMAL STUDIES

2.6.1 Mice

C57BL/6 mice and Balb/C athymic nude mice, 6 to 8 weeks old, were purchased from Charles River (Calco, Italy). FVB-NeuN transgenic mice (Guy et al., 1992) were bred in the animal facility of Istituto Nazionale Tumori, Milan. All animals were treated in accordance with institutional guidelines.

2.6.2 Assessment of tumourigenicity

Tumour cells in log-phase growth were trypsinised, washed twice in saline and resuspended in saline at the desired cell concentration (see Results for individual experiments). Mice were injected subcutaneously in the flank with 200 µl of the cell

suspension. The mice were examined daily until the tumour became palpable, whereafter the diameter, in two dimensions, was measured twice weekly using calipers. A tumour was considered to be present when a palpable mass > 0.2 cm was noted. Mice were killed when the tumour size was approximately 1.0 x 1.0 cm.

2.6.3 Vaccination of mice using irradiated tumour cells

Tumour cells suspensions were irradiated (20,000 rads) and resuspended in saline at the desired concentration (see Results for individual experiments). Mice were injected subcutaneously with 200 µl of the irradiated cell suspension twice with a 4-week interval. Mice that received a live tumour cell challenge were injected subcutaneously in the opposite flank of vaccination or intravenously 4 weeks after the second immunisation (see Results for individual experiments).

In some experiments mice received injection intraperitoneally with IL-12 (kindly provided by Dr. L Adorini, Roche Milano Ricerche, Milan. Italy), immediately after vaccination with irradiated cells and on days +1, +2 and +3 (150 ng/day diluted in PBS containing 100 µg/ml mouse serum albumin).

2.6.4 Collection and storage of mice sera

Mouse sera were obtained by bleeding from the retroorbital venous plexus. Blood was collected in sterile centrifuge tubes and allowed to clot at room temperature. After centrifugation for 10 min at 1500 rpm, the clots were discarded and the antisera transferred to fresh tubes and stored in small aliquots at -20°C.

2.6.5 Depletion of lymphocyte subsets

In vivo depletion of CD8+ cells was achieved by using an anti-CD8 monoclonal antibody as listed in Table 2.2 (protocol and antibodies were kindly provided by Dr.

Giovarelli, Department of Clinical and Biological Sciences, University of Turin). Animals were given 3 intraperitoneal injections of 1mg of antibodies over a period of 5 days. Control mice were injected with an isotype-matched irrelevant antibody.

In experiments of monitoring the passive transfer of immunity (Winn test), control splenocytes from mice *in vivo* depleted of CD8+ cells were subjected to passage over a nylon wool column (Berke et al., 1972; Julius et al., 1973) to obtain cell populations enriched in T-lymphocytes and depleted of adherent cells like B-lymphocytes, monocytes and macrophages. The cell suspension was slowly injected into a Uni-Sorb T&B Column (Nycomed Pharma As, Oslo, Norway) and the column incubated at 37°C for 1 hr. After incubation, the non-adherent T cells were eluted by washing through the column with three volumes of RPMI medium.

2.6.6 Cytokine production from popliteal lymph nodes cells.

In experiments to evaluate the patterns of cytokine release by T cells, mice were immunised s.c. into the right hind footpad with 5×10^6 irradiated cells in 50 µl of saline. Five days later, mice were sacrificed and popliteal lymph nodes were removed aseptically. Pools for each group were made, each containing the nodes of 2 or 3 animals. Lymphocytes were dissociated mechanically and cultured (2×10^5 cells per well) in 96-well flat-bottom plates precoated, or not, with 1 µg/well of anti-CD3 MAb at 37°C for 18 hr. Cytokine-containing supernatant was collected and tested for IFN-γ and IL-4 production by ELISA.

2.6.7 Statistical analyses

All statistical analyses were performed using a Graph Pad Prism computer program (GraphPad Software, Becton Dickinson). Statistical significance was considered to be $p < 0.05$.

In experiment to evaluate tumour growth, different groups of mice were compared by the logrank test, using the 'occurring event' as the time at which a tumour appeared. A tumour was considered present when a palpable mass > 0.2 cm was noted.

In experiment to evaluate the number of metastatic nodules and the cytokine production in different groups of mice statistical analysis was performed using the Unpaired t test.

Table 2.1 Cell lines used in the thesis

B16 murine melanoma	(Fidler, 1970)
B16.B78 murine melanoma	(Silagi et al., 1972)
NIH 3T3 murine fibroblast	(Pulciani et al., 1982)
GP+envAm12 retroviral packaging cell line	(Markowitz et al., 1988b)
GP+envE86 retroviral packaging cell line	(Markowitz et al., 1988a)
N202.1A murine mammary carcinoma	(Nanni et al., 2000)
N202.1E murine mammary carcinoma	(Nanni et al., 2000)
TT3 murine mammary carcinoma	(Nanni et al., 2000)
CHO-K1	ATCC

Table 2.2 Monoclonal antibodies and antisera used in the thesis

Antibody	**isotype**	**antigen**	**used**
HBT12 (Singh et al., 1992)	mouse	*M. tuberculosis* Ag38	IF: 10 μg/ml WB: 1μg/ml
H-2L^d/H-2D^b (Clone 28-14-8) Litton	mouse	Mouse MHC I (L^d, D^b, D^q, L^q)	IF: 10 μg/ml
H-2K^b (Clone 28.13.3) Litton	mouse	Mouse MHC I (K^b)	IF: 10 μg/ml
TIB126 ATCC	rat	Mouse pan MHC I-II	IF: 10 μg/ml
c-neu Ab-4 Calbiochem	mouse	Rat Neu	IF: 2.5μg/ml

TIB105 (Clone Lyt2) ATCC	rat	Mouse CD8	*In vivo* depletion
AZN-LAM antiserum (Schreurs et al., 1997)	rabbit	Mouse gp100	IF: 1:100 WB:1:3000 IPPT: 1:100
aPEP8 antiserum (Tsukamoto et al., 1992)	rabbit	Mouse TRP-2	IF: 1:50 WB:1:1000 IPPT: 1:50
agp70 antiserum (Traversari et al., 1989)	goat	MuLV gp70	IF: 1:100 IPPT: 1:100
atotal virus antiserum (Traversari et al., 1989)	goat	MuLV proteins	IF: 1:100 IPPT: 1:100
TA99 (Mattes et al., 1983)	mouse	Human TRP-1	IF: 10 μg/ml WB: 10 μg/ml
T311 (Chen et al., 1995)	mouse	Human Tyrosinase	IF: 10 μg/ml WB: 10 μg/ml IPPT: 10 μg/ml
CD3e (Clone145-2C11) Pharmingen	Armenian Hamster	Mouse CD3e	*In vitro* stimulation of T cells
IL-12 (Clone C17.8) (Wysocka et al., 1995)	Rat	Mouse IL-12 (p40/p70)	*In vivo* neutralization

Table 2.3 FITC-conjugated antibodies used for immunostaining for flow cytometry

(all purchased from Pharmingen)

Antibody	**Clone**	**Fluorochrome**	**dilution used**
Rat anti-mouse CD8	53-6.7	FITC	1μg/10^6 cells
Rat anti-mouse CD8	53-6.7	Cy-Chrome	1μg/10^6 cells

Rat anti-mouse CD4	H129.19	Cy-Chrome	1μg/10^6 cells
Rat anti-mouse IFN-γ	XMG1.2	FITC	0.5μg/10^6 cells
Rat anti-mouse IL-4	11B11	PE	0.5μg/10^6 cells
Mouse anti-mouse NK1.1	PK136	PE	1μg/10^6 cells

Table 2.4 FITC-conjugated secondary antibodies used for immunostaining for flow cytometry

FITC-conjugated antibody	**dilution used**	
goat anti-mouse IgG+IgM	10 μg/ml	Jackson ImmunoResearch
rabbit anti-goat IgG biotinylated + Streptavidin-FITC	10 μg/ml 20 μg/ml	Amersham Amersham
goat anti-rabbit IgG	10 μg/ml	Jackson ImmunoResearch
rat anti-mouse IgG1 (Clone A85-1)	1μg/10^6 cells	Pharmingen
rat anti-mouse IgG2a (Clone R19-15)	1μg/10^6 cells	Pharmingen
rat anti-mouse IgG2b (Clone R12-3)	1μg/10^6 cells	Pharmingen
rat anti-mouse IgG3 (Clone R40-82)	1μg/10^6 cells	Pharmingen
rat anti-mouse IgM (Clone R6-60.2)	1μg/10^6 cells	Pharmingen
rat anti-mouse IgA (Clone R5-140)	1μg/10^6 cells	Pharmingen
rat anti-mouse IgE (Clone R35-72)	1μg/10^6 cells	Pharmingen

Table 2.5 Horseradish peroxidase-conjugated secondary antibodies used in Western blot

(all purchased from Amersham)

Horseradish peroxidase -conjugated antibody	**dilution used**
Sheep anti-mouse immunoglobulins	0.1 µg/ml
Donkey anti-rabbit immunoglobulins	0.1 µg/ml
Sheep anti-rat immunoglobulins biotinylated + Streptavidin-Horseradish peroxidase	2.5 µg/ml 0.6 µg/ml

CHAPTER THREE

GENE TRANSFER AND EXPRESSION OF *AG38* GENE IN TUMOUR CELL LINES

3.1 CHOICE OF THE STRATEGY

Preliminary studies focused on creating an optimised strategy to express in an efficient and stable fashion the bacterial antigen in tumour cells.

The use of a conventional eukaryotic vector to express the entire mycobacterial gene, provided with its own signal peptide sequence, under the control of the CMV promoter resulted in a low and unstable expression of the bacterial product in tumour cells.

These studies led to the choice of a retroviral system to transduce the bacterial gene in tumour cells and to the use of a retroviral vector (pLXSN), modified to express the leader and transmembrane portion of the Nerve Growth Factor Receptor and the stop codon region of human IL-3 (pLTMSN).

As an initial hypothesis for the purposes of cloning using this vector, it was predicted that the modification of the vector would provide three advantages:

- the induction of cell surface expression of the bacterial antigen, thus allowing future detection and tagging of transduced cells;
- an increase in stability, gained by the presence of eukaryotic leader sequence, ensuring correct processing of the bacterial protein;
- the likely combination of peptides derived from the bacterial protein complexing with MHC class I molecules during transport through to the cell membrane, promoting the bacterial antigen immune recognition.

3.2 MODIFICATION OF pLXSN VECTOR AND CLONING OF *AG38* GENE

Plasmid pLTMSN, kindly provided by Dr M.P. Colombo (Istituto Nazionale Tumori, Milan), was obtained by modification of retroviral vector pLXSN. The modification, described below, was performed in the Dr. Colombo laboratory.

A fragment containing the first 843 bp of the nerve growth factor receptor (NGFR), lacking the intracellular domain, was cloned into the shuttle vector pGEM 5Zf+ (Promega); the extracellular portion of the NGFR was excised and replaced in-frame with a synthetic polynucleotide sequence containing three unique cloning sites (NotI, ClaI and EcoRV). The entire insert was designated pLTM. At the 3'-end, the last 28 bp of the human IL-3 sequence, containing the stop codon, were joined in-frame by replacement of the IL-3-encoding fragment with the pLTM fragment in the pBBG14 vector (British Biotechnology; Oxon, UK). The entire construct was excised from the plasmid and ligated into the retroviral vector pLXSN, to obtain the vector designated pLTMSN.

When I received this vector (empty) the DNA encoding residues 24-372 of the *Mycobacterium tuberculosis Ag38* gene was obtained by polymerase chain reaction (PCR) using plasmid pMS9-2 (kindly provided by Dr. Singh, GBF, Braunschweig, Germany) (Singh et al., 1992) as template and sense and antisense primers, which incorporate NotI and ClaI restriction sites, respectively. The sense primer corresponds to nucleotides 215-246 of the *Ag38* gene, thus excluding the signal peptide region of the *Ag38* gene; the antisense primer corresponds to nucleotides 1235-1267, thus excluding the stop codon region of the *Ag38* gene (Fig. 3.1). The amplified fragment was subcloned into the pLTMSN retroviral vector, in-frame with the NGFR signal peptide and transmembrane sequences, to obtain the pL*Ag38*TMSN vector (Fig. 3.2).

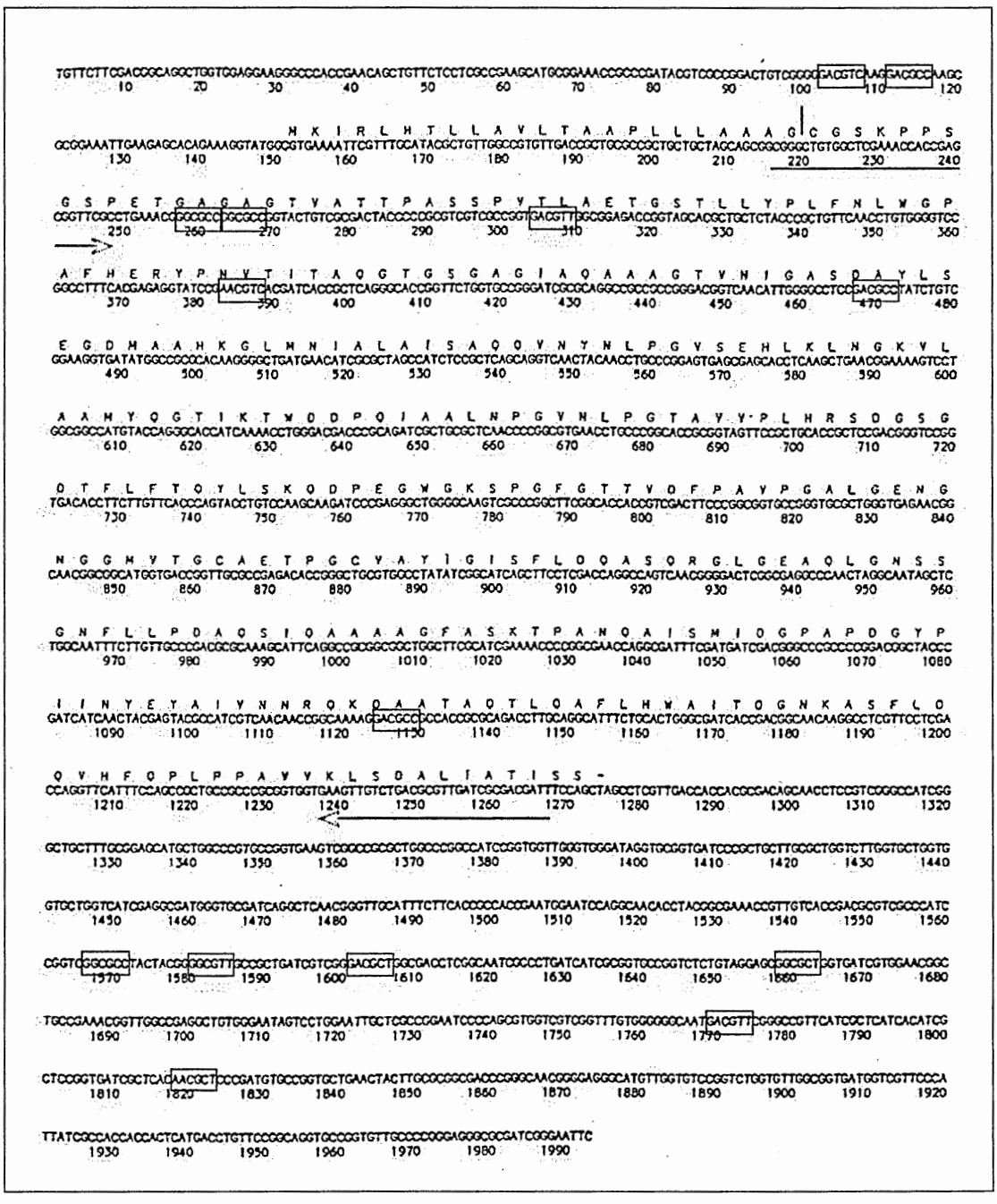
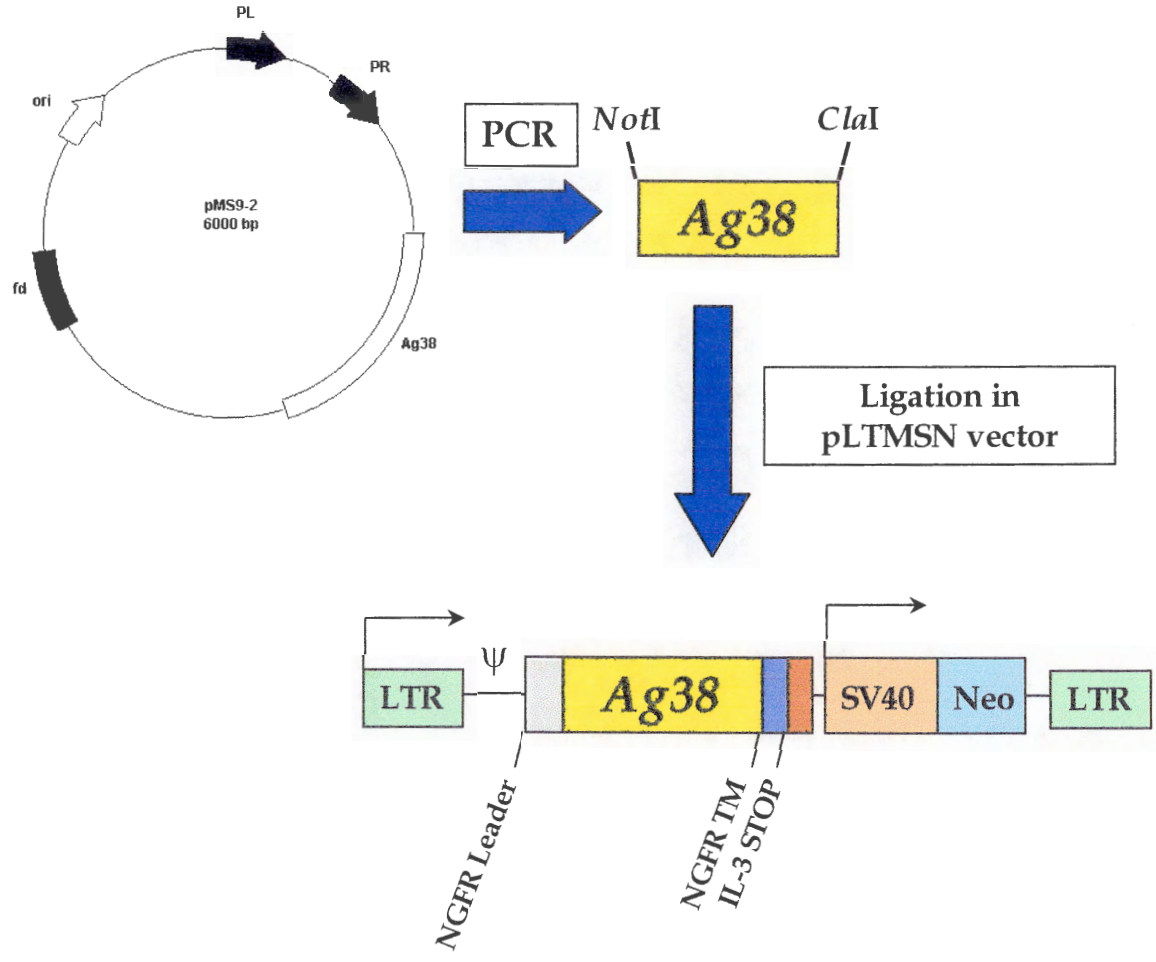


Fig. 3.1: *Ag38* gene sequence

Nucleotide sequences of *Ag38* gene and the deduced amino acid sequence of the protein product. Sense and antisense primers used to amplify the encoding region, excluding the signal peptide and the stop codon, are indicated by arrows (red). Putative processing site is indicated with a vertical bar (green); CpG motifs contained in the gene sequence are boxed (blue).



Ag38	primer
sense (nt 215-246)	5′-GCGGCCGCTGGCTCGAAACCACCGAGCGGTTCGCCTGAA-3′
antisense (nt 1235-1267)	5′-GCGGTGGTGAAGTTGTCTGACGCGTTGATCGCATCGATT-3′

Fig. 3.2: Cloning of *Mycobacterium tuberculosis Ag38* gene

Schematic diagram showing the strategy of cloning. The sequences of the sense and antisense primers used to amplify the gene are reported in the box.

3.3 GENERATION OF STABLE PRODUCER CELL LINES

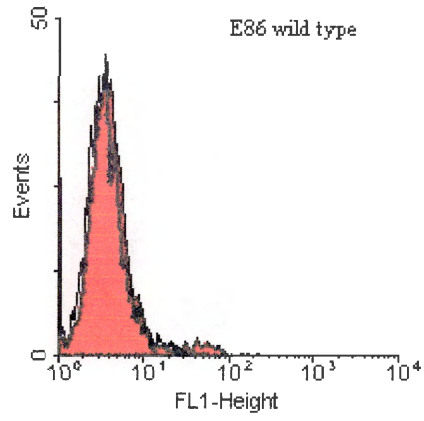
Retroviral particles transducing the pL*Ag38*TMSN vector were obtained by the infection technique as described in Materials and Methods, using the ampho-ecotropic packaging cell lines gp+AM12 and gp+E86 (Markowitz et al., 1988b; Markowitz et al., 1988a). G418-resistant individual colonies of producer cells were screened for cell surface Ag38 expression by FACScan analysis using anti-Ag38 MAb HBT12 (kindly provided by Dr. Singh, GBF, Braunschweig, Germany) (Singh et al., 1992) and characterised in terms of viral titre. The clone that showed the best characteristics, either as viral titre, or in terms of antigen expression, and the bulk culture were used to produce viral supernatant for tumour cells infections (Fig. 3.3).

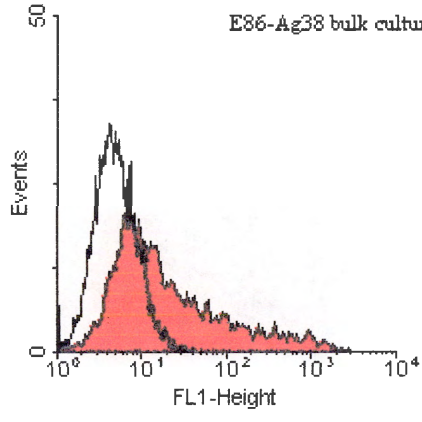
3.4 EXPRESSION OF THE *AG38* GENE IN MURINE MELANOMA CELL LINES

B16 murine melanoma cell line and its variant B16.B78 were infected with the pL*Ag38*TMSN viral supernatant.

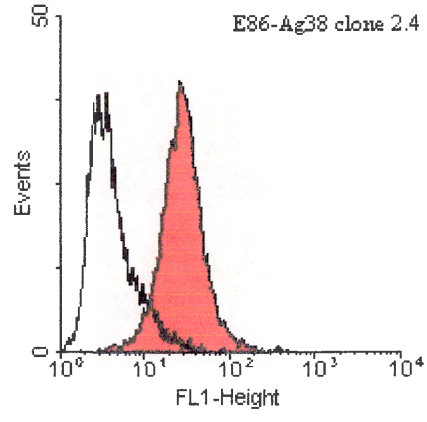
The murine melanoma B16.B78 is a clone of the C57BL/6 derived tumour B16, which expresses no detectable MHC class I antigen *in vitro*. On the contrary, low level expression of MHC class I molecules is detectable on B16 cells (Lollini et al., 1990). The level of MHC class I expression in the two different sublines was evaluated by FACScan analysis (Fig 3.4).

Infections were performed as reported in Materials and Methods. Five rounds of the same infection cycle were performed using the single clone as well as the bulk culture derived supernatants, to improve infection efficiency.





VIRAL TITRE:
2×10^7 cfu/ml



VIRAL TITRE:
3.4×10^6 cfu/ml

Fig 3.3: *Ag38* expression and viral titre of producer cell line

Expression of Ag38 protein on cell surface detected by FACScan analysis with HBT12 antibody and viral titres of the bulk culture and of selected clone 2.4. FACScan analysis with HBT12 antibody on wild type cells is shown on the top. Open areas indicate cells stained with secondary antibody alone.

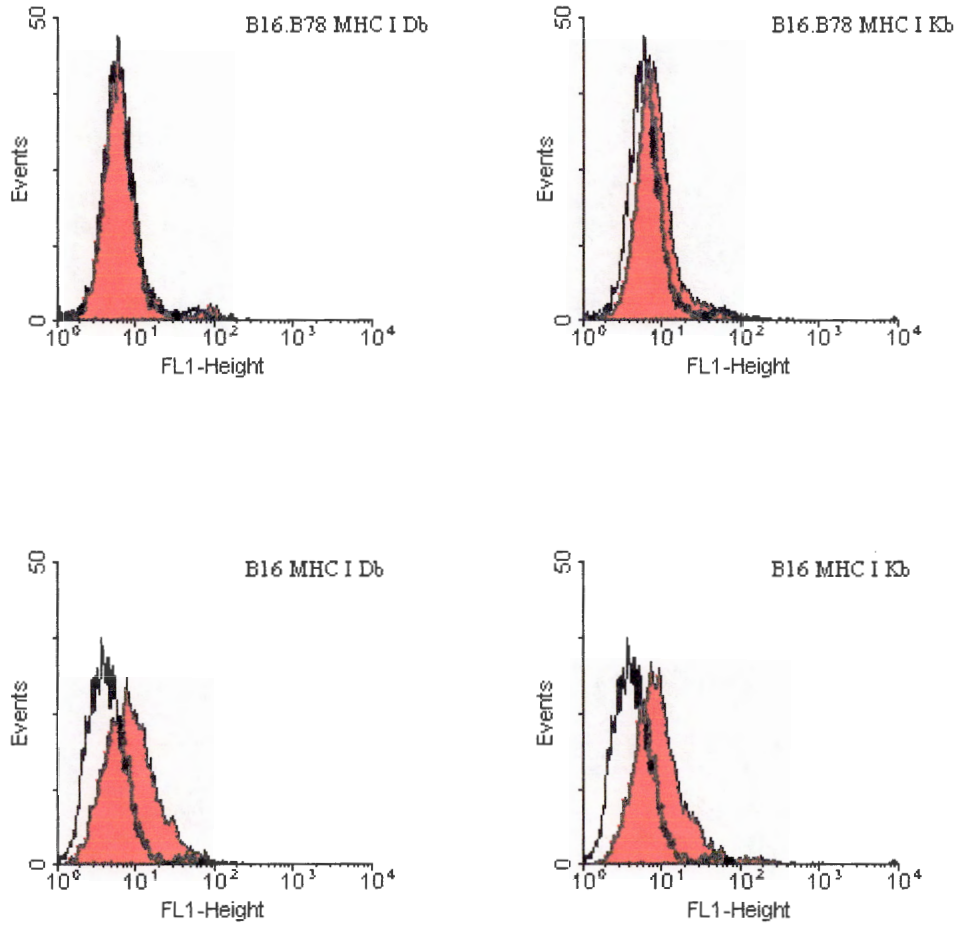


Fig. 3.4: FACscan analysis of MHC class I expression on murine melanoma lines

MHC class I expression on B16.B78 (top) and B16 (bottom) melanoma cells, detected by FACscan analysis with anti-mouse MHC class I Db and Kb antibodies (kindly provided by Dr. M. Rodolfo, Istituto Nazionale Tumori, Milan). Open areas indicate cells stained with secondary antibody alone.

Individual clones were selected in G418-containing medium and tested for bacterial antigen expression.

RT-PCR was performed to detect the expression of Mycobacterium tuberculosis *Ag38* transcript in transduced cells (Fig 3.5a).

Western blot analysis using MAb HBT12 failed to reveal any reactivity when performed on total cellular protein extract, but revealed two specific bands in the cell membrane protein extract. The two bands, characterised by a molecular weight of 47 kDa and 42 kDa, were supposed to correspond, respectively, to the unprocessed form, which contains both the leader and transmembrane sequences (123 and 117 nucleotides, respectively), and to the mature protein after cleavage of the leader sequence (Fig 3.5b).

The majority of transduced cells were positive for cell surface expression of the mycobacterial protein, as revealed by FACS analysis. Clones of B16.B78 and B16 cells showing the highest cell surface expression of Ag38 antigen are shown in Figure 3.6; B16.B78-*Ag38* clone 17 and B16-*Ag38* clone 7 were used in the subsequent experiments.

G418-resistant colonies recovered from B16 and B16-B78 melanoma cells transduced with the empty vector were obtained and used as controls (mock cells). Both wild-type and melanoma cells transduced with the empty vector showed no detectable mycobacterial product by RT-PCR, Western blot or FACScan analysis.

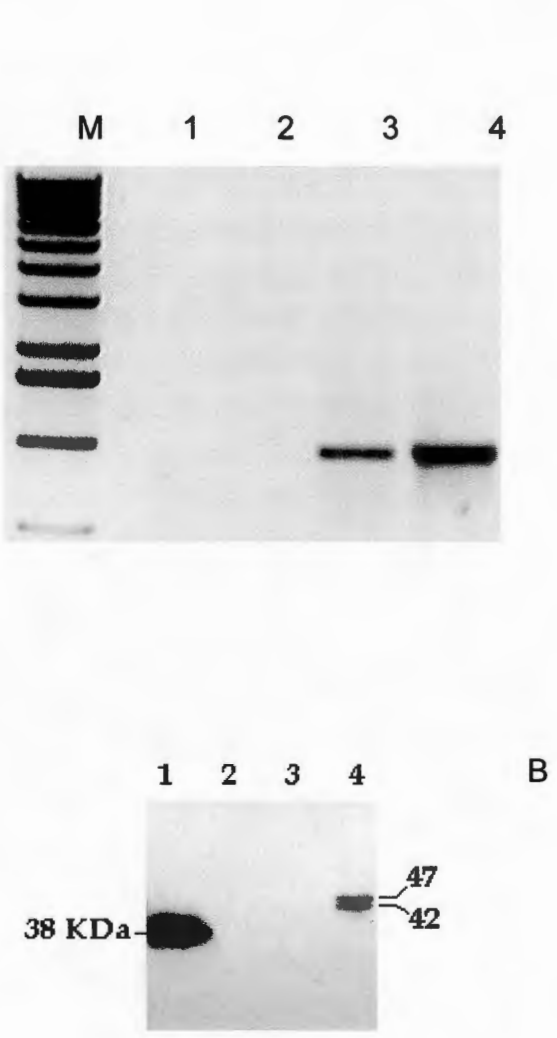


Fig. 3.5: *Ag38* gene expression in transduced melanoma cells

Expression of *Mycobacterium tuberculosis Ag38* gene in a representative clone of B16.B78 transduced cells.

A: Detection of *Ag38* mRNA by RT-PCR of total RNA from: B16-B78 wild-type melanoma cells (lane 1); B16-B78 cells transduced with pLTMSN vector (lane 2); B16-B78 cells transduced with pLAg38TMSN vector (lane 3). Lane 4, amplification of pLAg38TMSN vector; M, molecular weight marker (1 kb ladder);

B: Detection of Ag38 protein by Western blot analysis of membrane proteins extracted from: B16-B78 wild-type melanoma cells (lane 2); B16-B78 cells transduced with pLTMSN vector (lane 3); and B16-B78 cells transduced with pLAg38TMSN vector (lane 4). Lane 1, bacterial recombinant Ag38 protein.

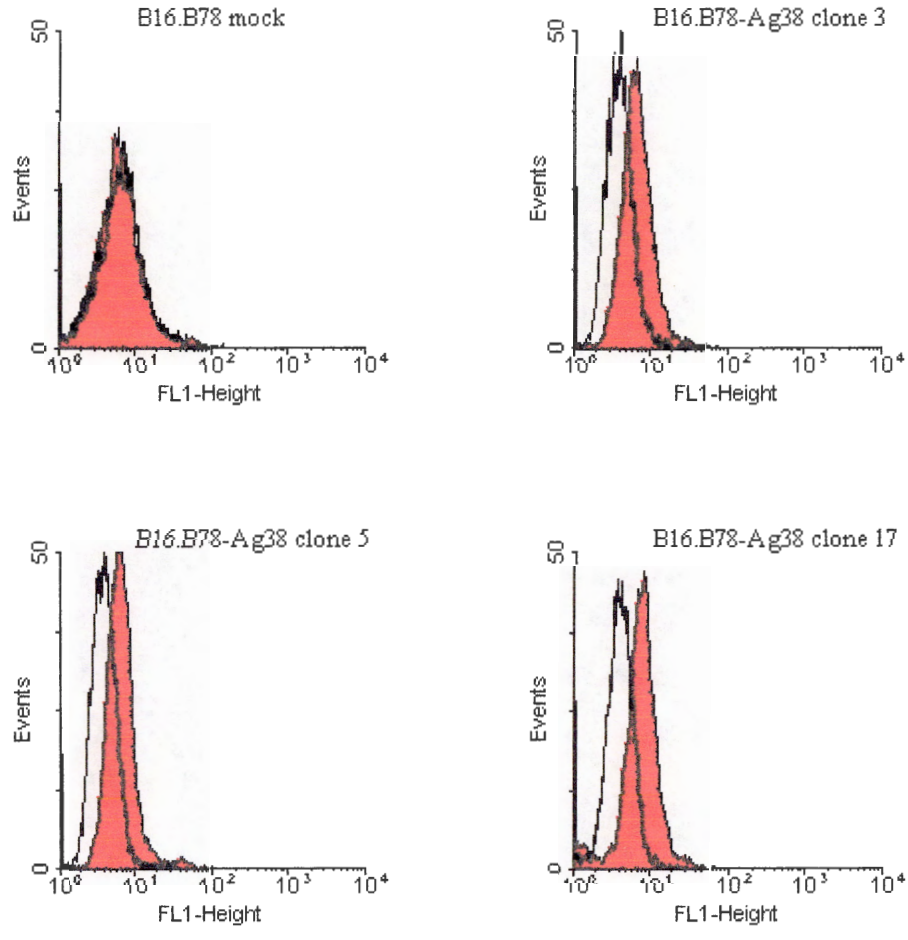


Fig. 3.6A: Cell surface expression of Ag38 protein on B16.B78 transduced cells

Detection of Ag38 protein by FACScan analysis on the surface of B16.B78 cells transduced with the pLTMSN vector (mock) or with the pLAg38TMSN vector. Clones showing the highest cell surface expression of Ag38 protein are represented. Open areas indicate cells stained with secondary antibody alone.

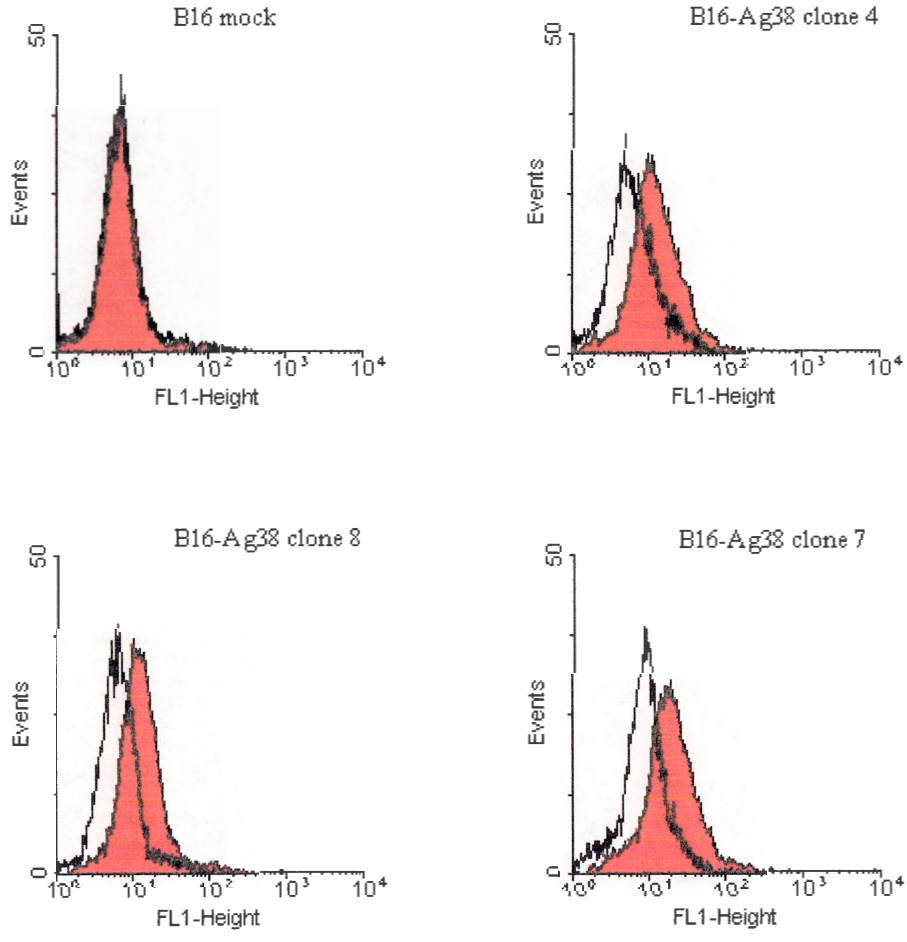


Fig. 3.6B: Cell surface expression of Ag38 protein in B16 transduced cells.

Detection of Ag38 protein by FACScan analysis on the surface of B16 cells transduced with the pLTMSN vector (mock) or with the pLAg38TMSN vector. Clones showing the highest cell surface expression of Ag38 protein are represented. Open areas indicate cells stained with secondary antibody alone.

3.5 EXPRESSION OF THE *AG38* GENE IN THE N202.1A MAMMARY CARCINOMA CELL LINE

Tumour cell line N202.1A was derived from a mammary carcinoma spontaneously grown in FVB-neuN transgenic mice (Kindly provided by Dr. P. Nanni, Institute for Cancer Research, University of Bologna, Italy). This cell line is characterised by expression of high levels of the HER2/neu oncogene on the cell membrane, as revealed by FACScan analysis with the specific antibody (Fig 3.7).

Furthermore, a high level of MHC class I molecules expression was detected by FACScan analysis with the specific antibody (Fig 3.7).

Ag38-transduced N202.1A cells were obtained by multiple rounds of infection cycle with pL*Ag38*TMSN viral supernatant and selected in G418-containing medium, as above described. Individual clones were tested for bacterial antigen gene expression by RT-PCR and FACScan analysis. Clones showing the highest cell surface expression of Ag38 antigen are shown in Figure 3.8; N202.1A-*Ag38* clone 4 was used in the subsequent experiments.

G418-resistant colonies recovered from N202.1A cells transduced with the empty vector were used as controls.

3.6 *IN VITRO* GROWTH OF CELLS EXPRESSING AG38 BACTERIAL ANTIGEN

In *in vitro* culture, cells expressing Ag38 protein had morphological appearances similar to the parental cells. No differences in growth between the parental and the Ag38-expressing cells were observed in the three different lines. Only minimal differences in

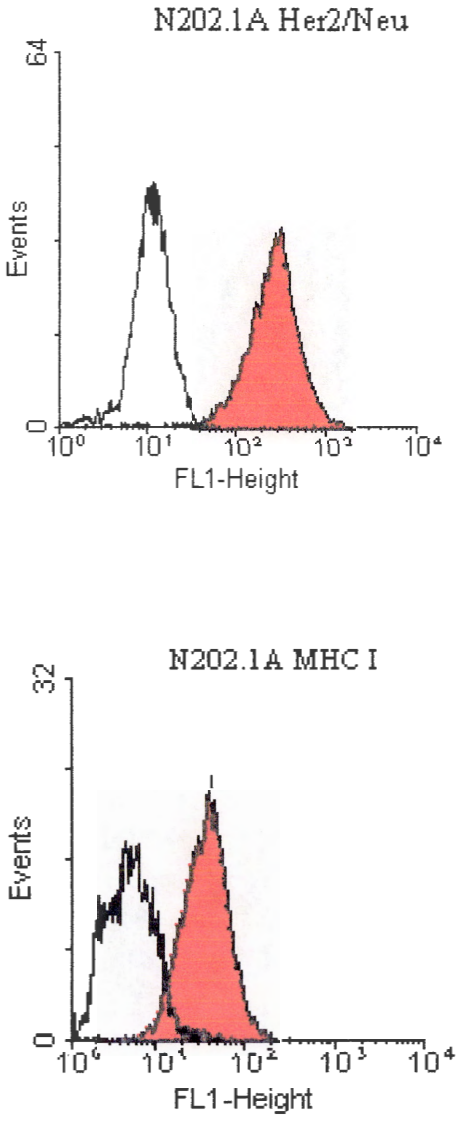


Fig. 3.7: FACScan analysis of Her2/Neu and MHC class I expression on N202.1A cell line

Characterisation of N202.1A cell line as Her2/Neu oncogene (top) and MHC class I (bottom) expression, detected by FACscan analysis with the specific antibodies (c-neu-Ab4 and TIB126, respectively). Open areas indicate cells stained with secondary antibody alone.

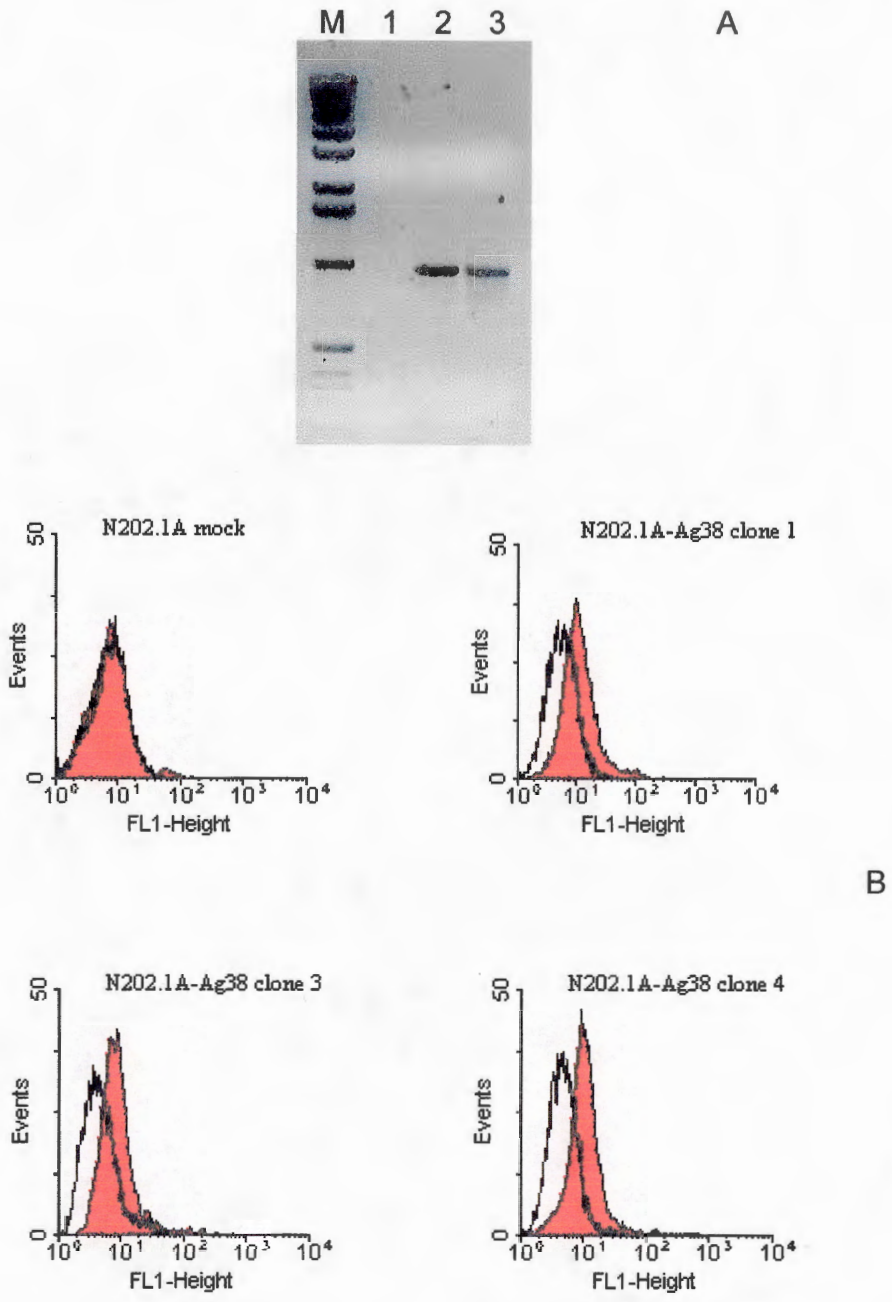


Fig. 3.8: Expression of *Ag38* gene in N202.1A transduced cells.

A: Detection of *Ag38* mRNA by RT-PCR of total RNA from: N202.1A cells transduced with pLTMSN vector (lane 1); N202.1A cells transduced with pLAg38TMSN vector (lane 2); and amplification of pLAg38TMSN vector (lane 3). M, molecular weight marker (1 kb ladder).

B: Detection of Ag38 protein by FACScan analysis on the surface of N202.1A cells transduced with the pLTMSN vector (mock) or with the pLAg38TMSN vector. Clones showing highest expression are represented. Open areas indicate cells stained with secondary antibody alone.

size of the cells were detected in the N202.1A cell line, in which Ag38-expressing cells appeared slightly larger than parental cells.

Stable expression of Ag38 protein was observed in all tumour cell lines after several *in vitro* passages. No modulation in MHC class I expression was detected in any cell lines and no modulation in *HER2/neu* oncogene expression was revealed by FACscan analysis in N202.1A-*Ag38* cells (data not shown).

CHAPTER FOUR

MODULATION OF THE IMMUNE RESPONSE AGAINST MELANOMA BY VACCINATION WITH *Ag38*-TRANSDUCED TUMOUR CELLS IN C57BL/6 MICE

4.1 TUMOURIGENICITY OF *AG38*-TRANSDUCED MELANOMA CELLS

As a first approach, the efficacy of a cancer vaccine expressing the Ag38 bacterial antigen was evaluated using the B16.B78 melanoma cell line. The *in vivo* growth of parental and Ag38-expressing B16.B78 cells was investigated by subcutaneous injection of $2.5x10^5$ cells in the right flank. Mice were monitored for the development of a tumour, which was considered to be present when a mass measuring greater than 0.2×0.2 cm was detected. Figure 4.1 shows the results of a representative experiment comparing the growth of parental and *Ag38*-transduced cells in syngeneic immunocompetent mice. All 5 mice injected with B16-B78 cells developed tumours within 9 to 21 days (mean 13.8) and had a mean survival time of 36.6 days. Necroscopy revealed lung metastases in all of these mice. By contrast, 3 out of 5 mice injected with $2.5x10^5$ transduced melanoma cells developed tumours and these mice showed a delay in appearance of the tumour (mean 30.6 days) that resulted in an increase in survival time (mean 52.3 days); no metastases were observed in the lungs, liver, kidneys or peritoneal cavity. Two different 38-kDa transduced clones also showed no spontaneous metastasis (not shown), making it unlikely that the *Ag38* gene was inserted into a gene involved in metastasis. However the absence of spontaneous metastasis cannot be definitively attributed to the expression of *Ag38* since B16 melanoma cell clones are reportedly heterogeneous with respect to metastatic potential (Poste et al., 1980).

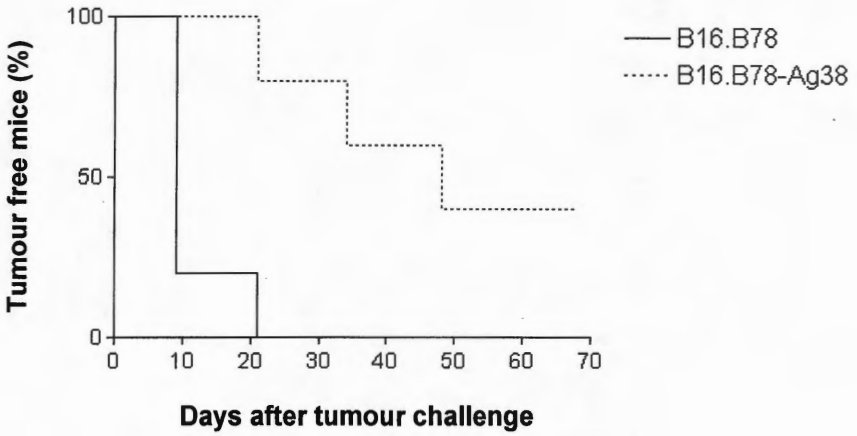


Fig. 4.1: Tumourigenicity of *Ag38*-transduced B16.B78 cells

Percentage of tumour free mice after challenge with $2.5x10^5$ parental B16.B78 cells ($n = 5$, solid line) or $2.5x10^5$ Ag38-expressing B16.B78 cells ($n = 5$, dotted line).

4.2 PROTECTIVE IMMUNITY AGAINST PARENTAL CELLS BY VACCINATION WITH *AG38*-TRANSDUCED MELANOMA CELLS

Analysis into whether vaccination with irradiated *Ag38*-transduced melanoma cells protects mice against a subcutaneous or intravenous challenge with parental cells was used to determine the immunogenicity of the transduced clones.

The same immunisation protocol was used in all experiments. 10^6 transduced or parental irradiated melanoma cells were injected s.c. twice, with a 4-week interval between injections, in the left flank of syngeneic mice. Four weeks after the second immunisation, the ability of *Ag38*-expressing melanoma cells to elicit a protective systemic immunity was evaluated by two approaches:

- Mice were challenged subcutaneously in the right flank with 2.5×10^5 viable parental cells and then monitored for the development of a tumour.
- Mice were challenged intravenously with 5×10^5 viable parental cells and evaluated for the number of lung metastases or survival time.

4.2.1 Protection induced by vaccination with Ag38-transduced cells against subcutaneous tumour

Figure 4.2 shows the result of a subcutaneous challenge experiment performed with B16.B78 transduced cells. All of 9 naive mice and 8 out of 10 mice immunised with wild-type B16.B78 melanoma cells developed tumours, whereas significant protection ($p<0.01$) was observed in mice immunized with the *Ag38*-transduced B16.B78 melanoma cells, since only 2 out of 10 mice developed tumours. Moreover, in these mice, the onset of tumour development as well as the mortality were delayed in comparison with non-immune and wild-type melanoma-immunised animals.

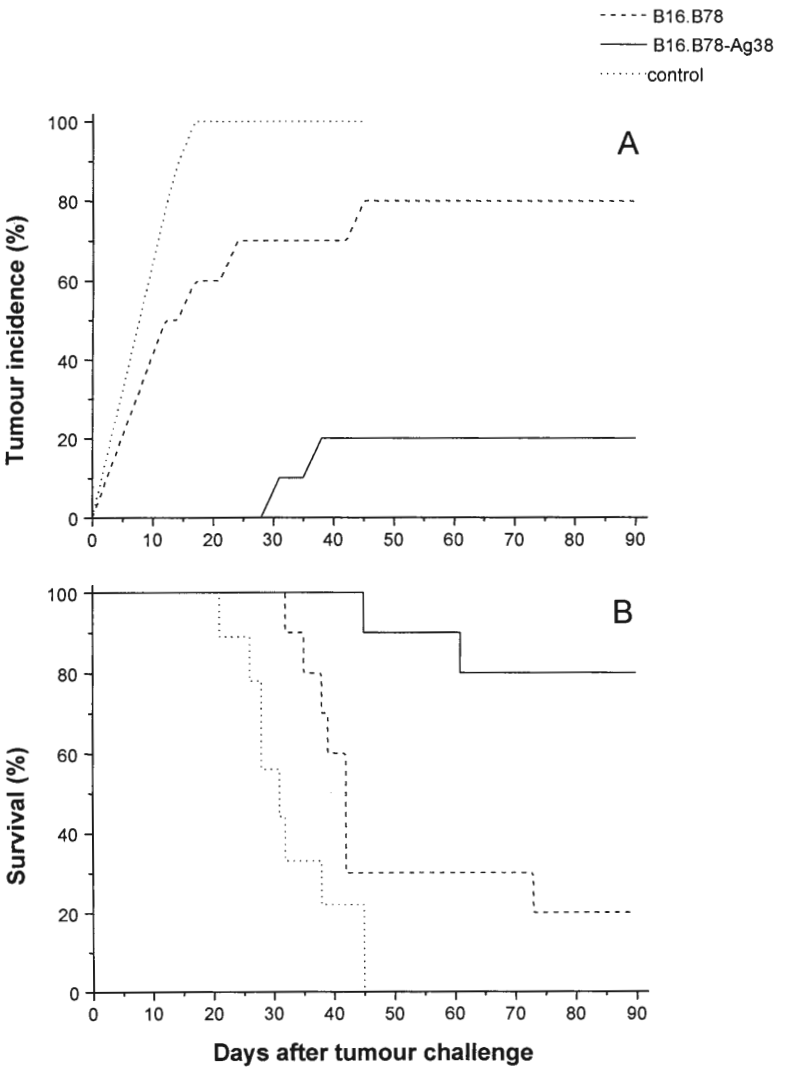


Fig 4.2: Protection against subcutaneous tumour by vaccination with *Ag38*-transduced melanoma cells

Percent tumour incidence (**A**) and overall survival (**B**) of mice either non-immunized (n = 9, dotted line) or immunised with irradiated wild-type B16-B78 (n = 10, dashed line) or with irradiated transduced B16-B78 melanoma cells (n = 10, solid line). Mice were challenged with 2.5×10^5 wild-type B16-B78 melanoma cells.

Since wild-type melanoma cells are highly tumourigenic and poorly immunogenic, these data suggest that the expression of the 38-kDa antigen in transduced cells elicits a strong immune response against the tumour.

Endogenous expression of Ag38 appears to be essential for protection, since C57BL/6 mice immunised with the purified 38-kDa protein developed tumours with no significant difference in respect to non-immunised controls, when challenged with wild-type melanoma cells (see chapter 6).

4.2.2 Protection induced by vaccination with Ag38-transduced cells against experimental lung metastases

Figure 4.3 shows the result of intravenous challenge experiments using B16.B78 transduced cells. In a first experiment the number of experimental lung metastases induced by i.v. inoculation of parental B16.B78 cells was evaluated in a group of mice immunised with irradiated *Ag38*-transduced B16.B78 cells. Inhibition of experimental metastasis development was observed in mice immunised with transduced cells, as indicated by the absence, or the low number, of lung metastases at 4 weeks after challenge, as compared to control mice (A).

Two experiments were then performed using the same challenge protocol to evaluate the effect of immunisation on survival time. In the first experiment (B), the 6 mice immunised with *Ag38*-transduced cells survived significantly longer than the 6 non-immunised control mice ($p=0.0005$), which all developed lung metastases and died within 31 days after tumour inoculation. Two out the 6 immunised mice were still alive at the end of the observation period (90 days). In the second experiment (C), a significant increase in survival time ($p=0.0001$) was observed in the 8 immunised mice versus the 8 control mice, and 2 animals were still alive after 150 days.

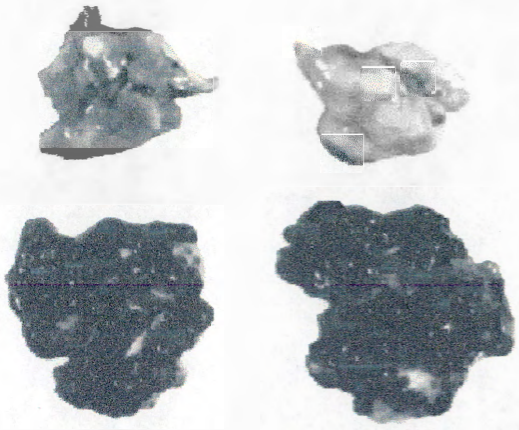


Fig 4.3A: Protection against experimental lung metastases by vaccination with *Ag38*-transduced melanoma cells: evaluation of metastases number

Lung metastases from mice immunised with irradiated *Ag38*-transduced B16.B78 melanoma cells (top) and from non-immunised mice (bottom), sacrified 4 weeks after i.v. challenge with 5×10^5 wild-type B16.B78 melanoma cells.

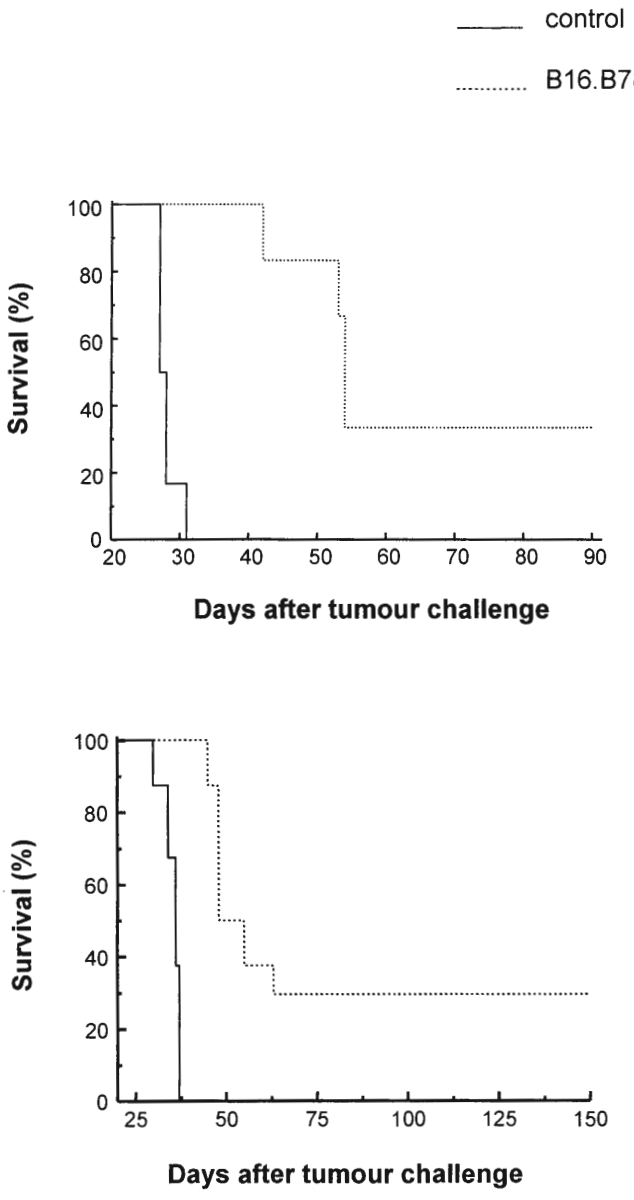


Fig. 4.3B: Protection against experimental lung metastases by vaccination with *Ag38*-transduced melanoma cells: evaluation of survival time

Two representative experiments showing the overall survival of mice immunised with irradiated *Ag38*-transduced B16-B78 melanoma cells (dotted line) or non-immunised mice (solid line) after i.v. challenge with $5x10^5$ wild-type B16-B78 melanoma. $n = 6$ per group in the experiment represented in the top and $n = 8$ per group in the experiment represented in the bottom.

These data indicate that vaccination with murine melanoma cells transduced with the 38-kDa Mycobacterium gene induces a significant inhibition of lung metastases of i.v.-injected parental tumours, leading to prolonged survival.

The efficacy of vaccination with B16 melanoma cell line transduced with the *Ag38* gene was evaluated for its protection against experimental metastases. Unlike the B16.B78 cell line, B16 cells express a low level of MHC class I molecules.

Results similar to those obtained with the B16.B78 variant were observed. Indeed, after i.v. challenge with $5x10^5$ untransduced parental B16 melanoma cells, 4 out of the 6 mice vaccinated with *M. tuberculosis Ag38*-transduced B16 cells showed an increase in survival time (mean 50.8 ± 5.8) as compared to the control group (mean 27.2 ± 0.4). The remaining 2 mice survived until the 90th day with no metastases ($p<0.01$). Therefore, irrespective of MHC context, the endogenous expression of Ag38 antigen in melanoma cells induces a significant inhibition of lung metastases of i.v.-injected parental tumours.

4.3 TH1- AND TH2-TYPE CYTOKINE SUBSETS ACTIVATED BY VACCINATION

Based on increasing evidence that suggests the importance of the Th1-type response in the control of tumour growth, the Th1- and Th2-types of reactions in mice vaccinated with *Ag38*-transduced cells were evaluated.

Mice were immunised s.c. into the right hind footpad with i) 5×10^6 irradiated wild-type melanoma cells, ii) 5×10^6 irradiated *Ag38*-transduced cells, and iii) 5×10^6 irradiated wild-type melanoma cell plus 3 μg Ag38 purified protein. Five days later, mice were

sacrified and popliteal lymph nodes were removed aseptically. Pools for each group were made, each containing the nodes of 2 or 3 animals. Lymphocytes were dissociated mechanically and cultured in plates precoated, or not, with anti-CD3 MAb at 37°C for 18 h. The result of a representative experiment is reported in Table 4.1. Analysis of cytokine levels in the culture supernatants revealed an increase in IFN-γ levels in mice injected with B16.B78 transduced cells compared to mice injected with irradiated wild-type melanoma cells and to mice injected with irradiated wild-type melanoma cells plus the 38-kDa purified antigen. In contrast, secreted IL-4 levels were higher in mice treated with wild-type B16.B78 plus purified 38-kDa protein than in mice injected with transduced cells.

These data suggest that the endogenous expression of Ag38 antigen in tumour cells induces a preferential induction of a Th1 response.

A similar trend, which confirms this preferential pattern, was observed using the B16 cell line to perform the same experimental protocol. Indeed, lymphocytes obtained from popliteal lymph nodes of mice immunised with transduced cells showed a ratio of 12.5±5.8 of IFN-γ:IL-4 production versus a ratio of 4.8±0.6 observed in mice immunised with wild type cells.

Injected antigen	Cytokine production (pg/ml)*	
	IL-4	IFN-γ
B16.B78	5.6 ± 0.7	8.2 ± 1.1
B16.B78-*Ag38* transduced	<5	32.0 ± 7.5
B16.B78+Ag38 protein	13.6 ± 2.0	<5

Table 4.1: Production of IL-4 and IFN-γ by popliteal lymph nodes of vaccinated mice.

Data refer to a representative experiment performed in triplicate and are expressed as mean±SD obtained by subtracting the cytokine levels in the supernatant of lymphocytes cultured in the absence of anti-CD3 antibody.

IL-4/IFN-γ ratio between: B16 transduced to B16 p=0.0013; B16 transduced to B16+Ag38 p=0.0109 (Unpaired t test).

4.4 LYMPHOCYTES SUBSETS INVOLVED IN MEDIATING PROTECTIVE IMMUNITY

Because of the importance of the CD8-mediated response in tumour rejection, the role of this subpopulation was investigated in mice depleted of $CD8^+$ T lymphocytes, using the specific monoclonal antibody TIB 105.

Figure 4.4A shows FACS analysis of splenocytes from mice subjected to the depletion protocol to remove $CD8^+$ lymphocytes and demonstrates depletion of the appropriate subset.

Depleted mice were vaccinated with *Ag38*-transduced melanoma cells and i.v. injected with parental melanoma cells, using the same vaccination-challenge protocol reported above. Non-depleted mice immunised with *Ag38*-transduced cells or non immunised mice were injected with parental cells and used as control groups. As shown in Figure 4.4B, CD8 depletion induced a decrease, although not significant, of survival time with respect to non-depleted immunised mice.

4.5 SUMMARY

Expression of *Mycobacterium tuberculosis* Ag38 antigen reduced the tumourigenicity of murine melanoma cells in immunocompetent mice.

Vaccination with *Ag38*-transduced cells induced a significant protection of mice against both subcutaneous and intravenous challenge with parental melanoma cells, indicating that the presence of mycobacterial protein greatly enhances immunological recognition of structures expressed by parental cells.

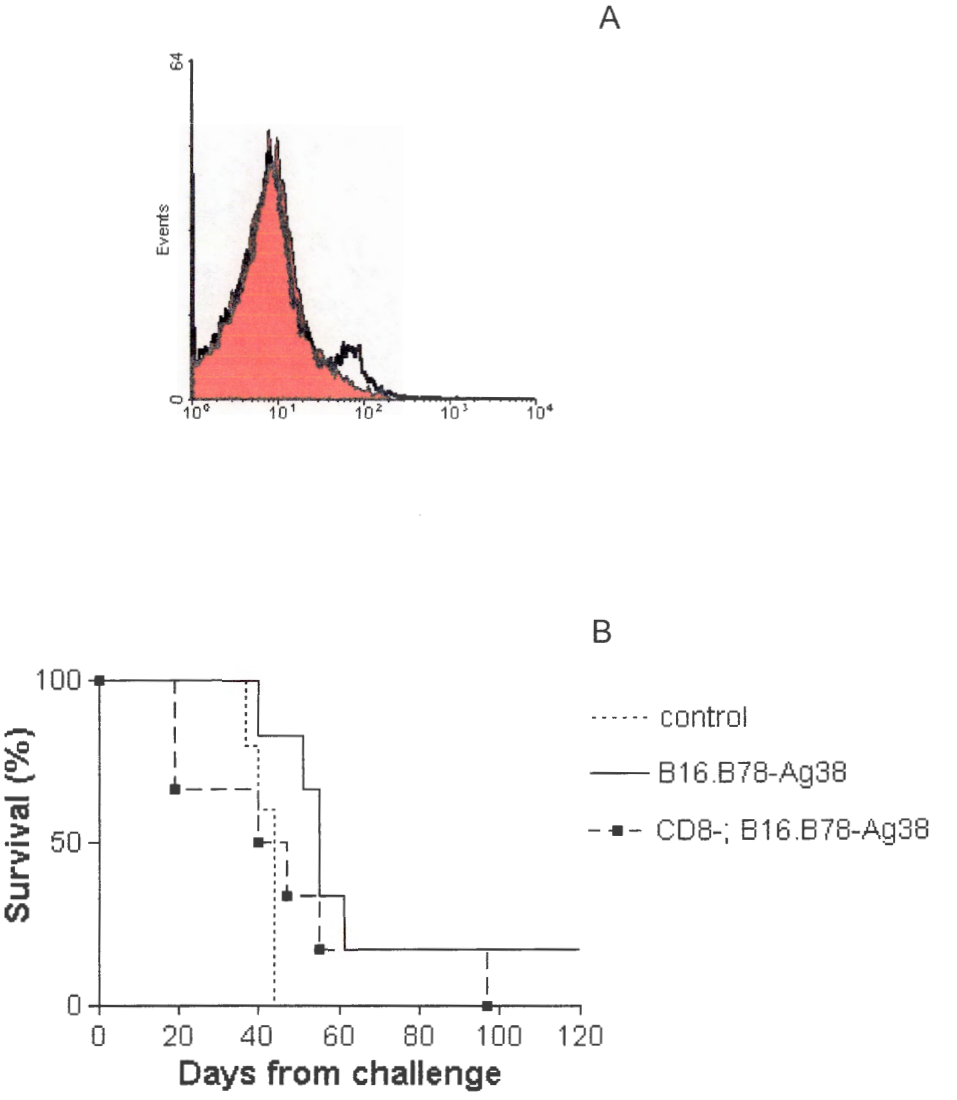


Fig. 4.4: Protection against experimental lung metastases in CD8-depleted mice

A: FACScan analysis with FITC-anti-CD8 antibody on splenocytes from mice depleted of $CD8^+$ T lymphocytes (filled) or non-depleted control mice (overlay).

B: Overall survival after i.v. challenge with 5×10^5 wild-type B16.B78 cells of non-depleted control mice (n = 5), non-depleted and immunised with B16.B78-*Ag38* cells mice (n = 6) CD8-depleted and immunised with B16.B78-*Ag38* cells mice (n = 6).

Comparison of Th1 and Th2 responses in immunised mice revealed a preferential Th1 response when the Ag38 protein was endogenously expressed.

Similar protection and response to vaccination was observed using both *Ag38*-transduced B16.B78 and B16 cell line variants, suggesting that the mechanism of protection is independent from MHC class I expression level in tumour cells. CD8 depletion experiments revealed that tumour protection was mediated by the involvement of different immune cell subsets.

CHAPTER FIVE

RESPONSE AGAINST MELANOMA TUMOUR ANTIGENS

5.1 HUMORAL RESPONSE

The immunological effectors involved in the response against parental melanoma cells, elicited by *Ag38*-transduction, were investigated.

As a first approach, the induction of an humoral response by vaccination with *Ag38*-transduced melanoma cells was analysed. The presence of antibodies directed against antigens expressed on melanoma cell surface in sera obtained from mice vaccinated with irradiated cells was evaluated by flow cytometry analysis.

As shown in Figure 5.1, sera obtained from mice vaccinated with *Ag38*-transduced cells showed very low titres of antibodies reacting with parental cells. Indeed, no significant differences in reactivity against parental cells were observed between sera obtained from mice vaccinated with *Ag38*-transduced cells and sera from non-vaccinated mice or mice vaccinated with non-transduced cells.

Furthermore, as shown in Fig. 5.2, no specific response against Ag38 mycobacterial protein was induced by vaccination with transduced cells.

The low serological response against melanoma cells was in keeping with the hypothesis that vaccination with tumour cells that endogenously express the mycobacterial protein induced a preferential Th1 response.

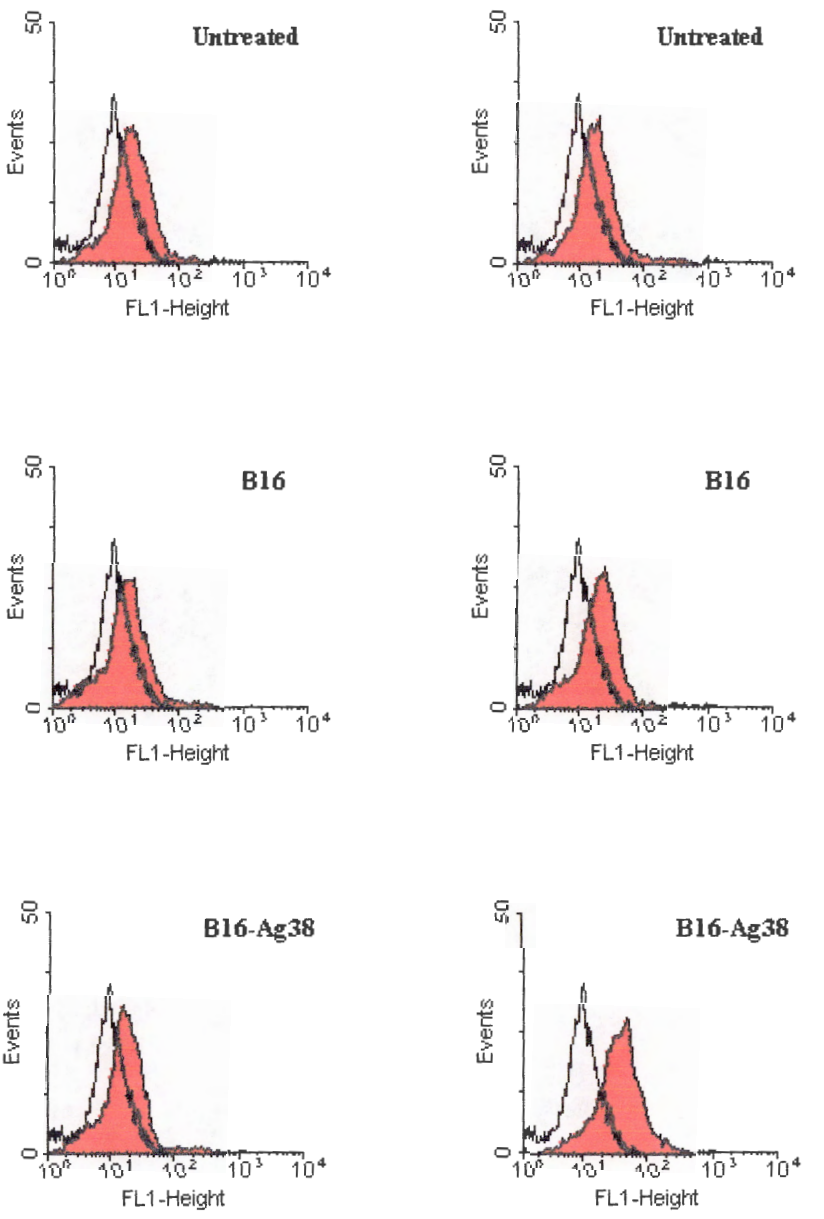


Fig. 5.1: Humoral response against parental melanoma cells after vaccination

Reactivity against melanoma antigens in some representative serum samples from not vaccinated mice (untreated), mice vaccinated with non-transduced cells (B16) and mice vaccinated with *Ag38*-transduced cells (B16-Ag38). FACScan analysis was performed on B16 cells, incubated with serum diluted 1:50 and stained by an indirect method using goat FITC-anti-mouse IgG+IgM. Open areas indicate cells stained with secondary antibody alone

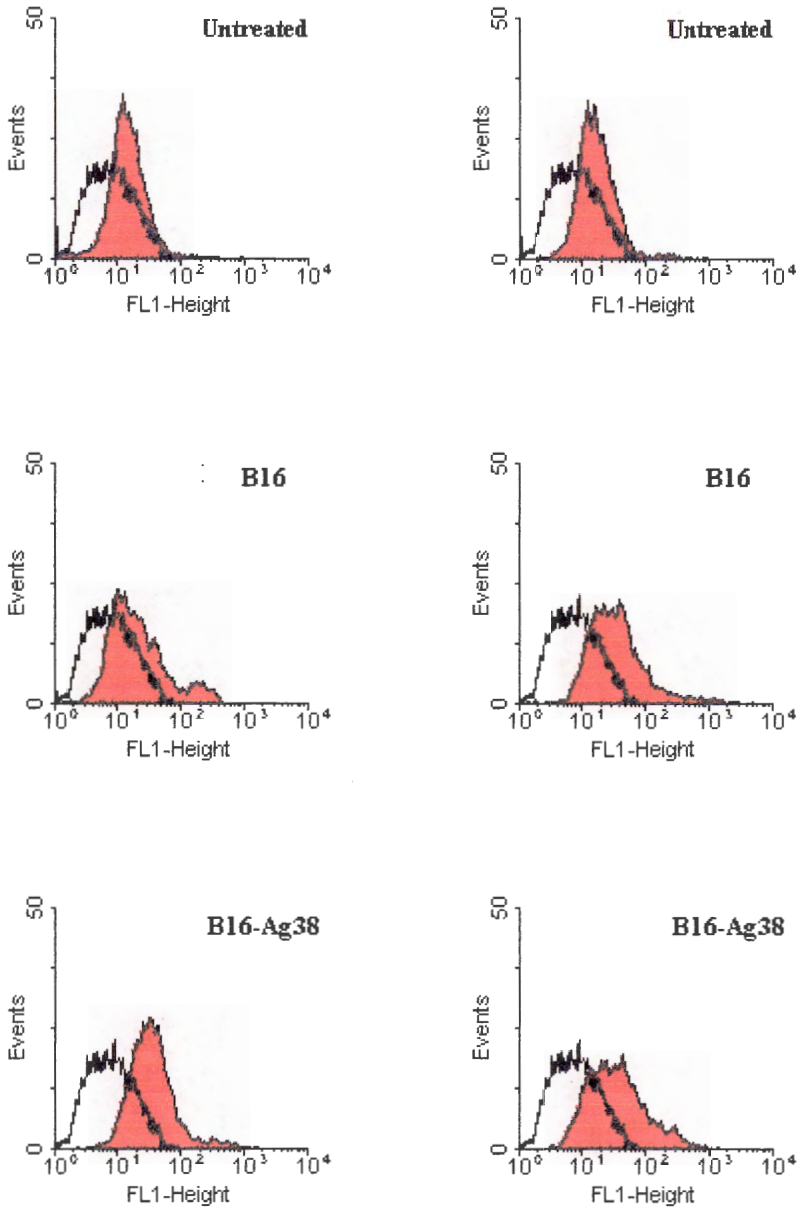


Fig. 5.2: Humoral response against *Ag38*-transduced melanoma cells after vaccination

FACScan analysis performed on Ag38-expressing melanoma cells with some representative serum samples obtained from not vaccinated mice (untreated), mice vaccinated with non-transduced cells (B16) and mice vaccinated with *Ag38*-transduced cells (B16-Ag38). Dilution of sera and cell staining were performed as in figure 6.1. Open areas indicate cells stained with secondary antibody alone

Interestingly, mice vaccinated with *Ag38*-transduced cells, which resulted partially or completely protected from the intravenous challenge with parental melanoma, displayed very high titres of antibodies against melanoma cells. A representative serum sample from a protected mouse is shown on figure 5.3.

Isotype analysis of these antibodies revealed the presence of mainly IgG2a immunoglobulins (Fig. 5.4). Since this class represents the main IFN-γ-dependent isotype in mice, it is likely that the humoral response observed after challenge with viable cells is related to the presence of mycobacterial-activated Th1 lymphocytes against melanoma-associated antigens.

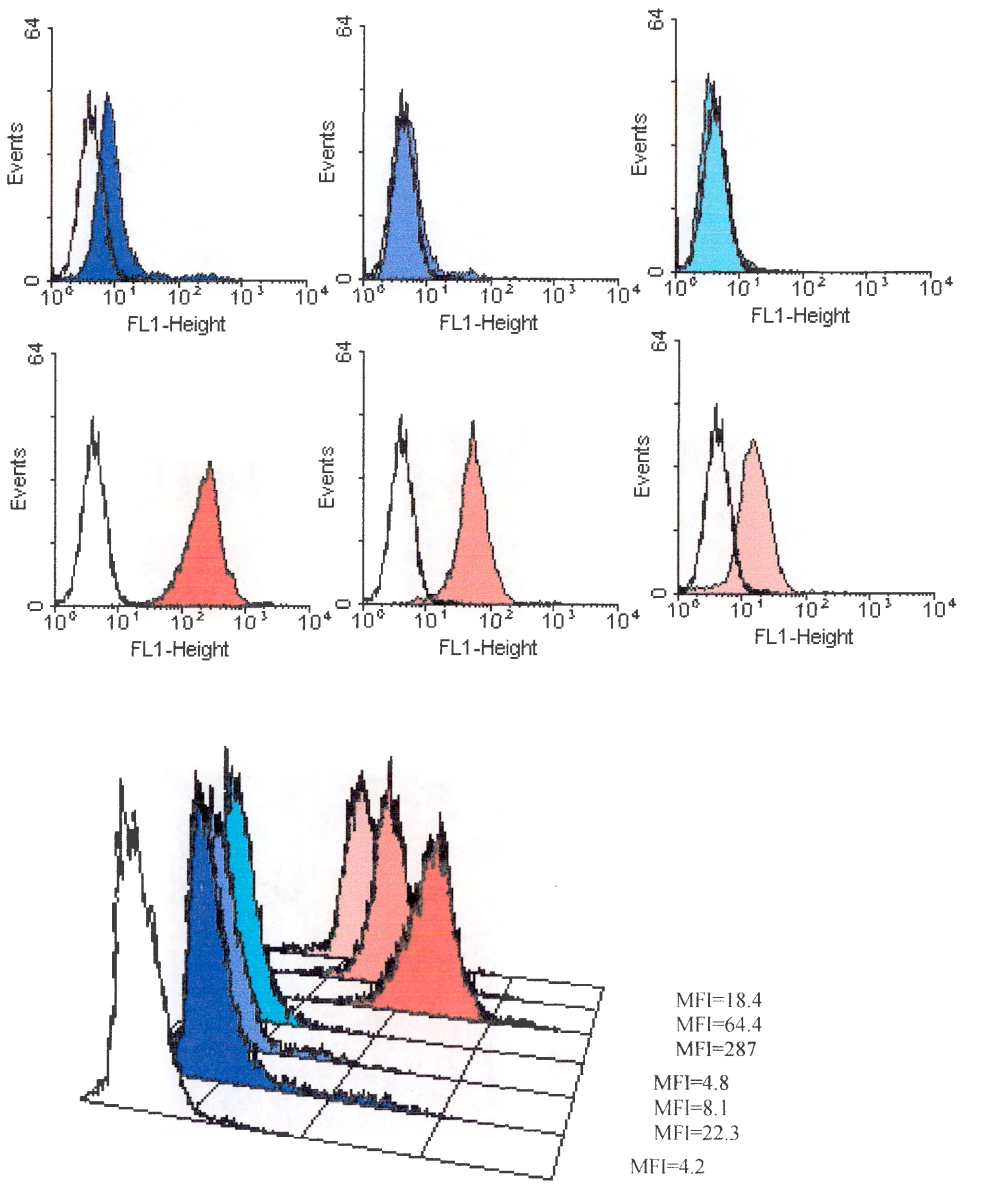


Fig. 5.3: Humoral response against parental melanoma cells in mice protected from tumour challenge

FACS analysis of a representative serum sample obtained from a mouse vaccinated with *Ag38*-transduced cells and protected from tumour challenge. Reactivity against B16 cells was evaluated before (upper; blue color) and after (down; red color) tumour challenge at three different dilutions. Open areas indicate cells stained with secondary antibody alone (goat FITC-anti-mouse IgG+IgM). MFI = mean fluorescence intensity (log).

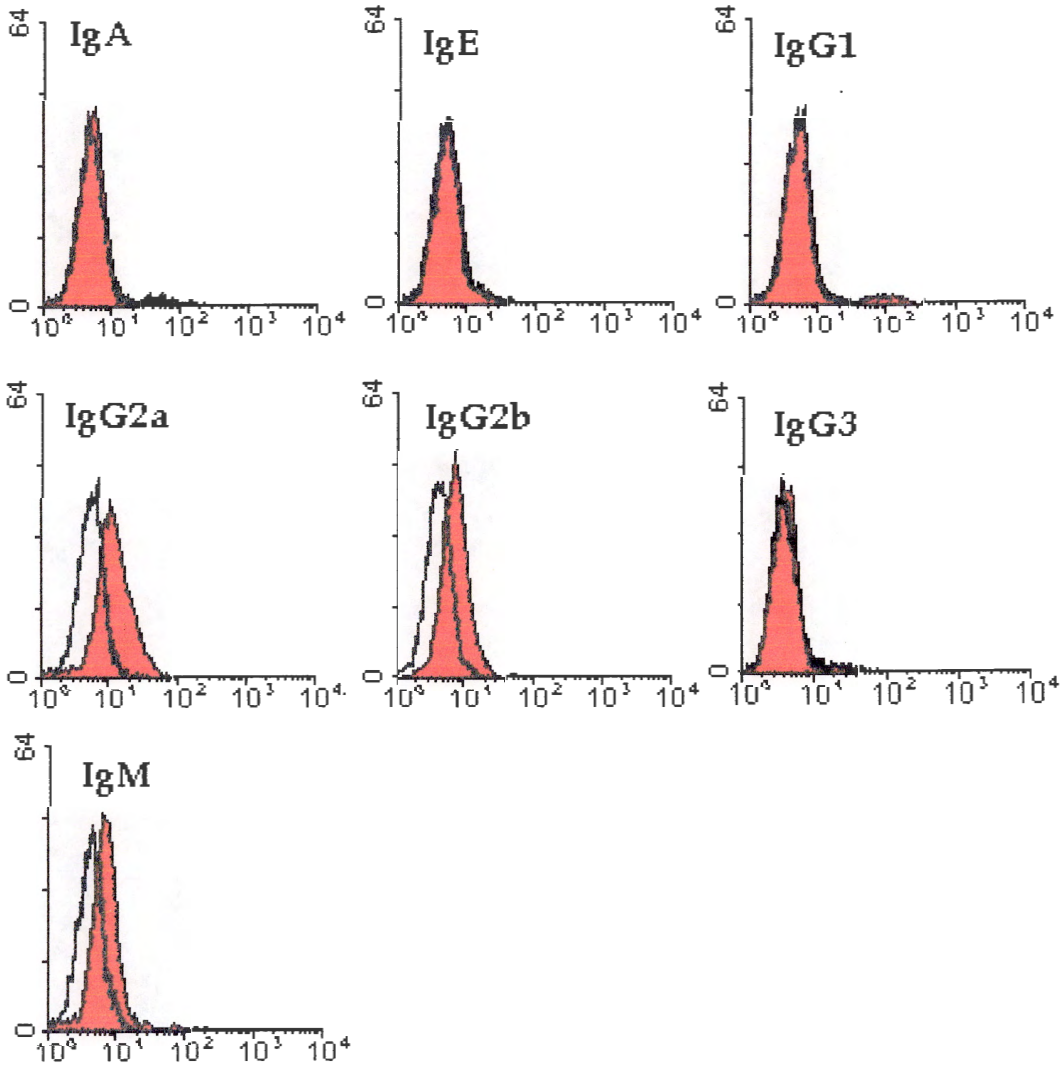


Fig. 5.4: Isotype analysis of anti-melanoma cells antibodies

Antibody isotype analysis performed on B16 cells incubated with post-challenge serum diluted 1:100 and stained using rat FITC-anti-mouse IgA, IgE, IgG1, IgG2a, IgG2b, IgG3, or IgM.

5.2 ANTITUMOUR ACTIVITY OF ANTIBODIES

In view of the increase in antibody titre against parental melanoma cells, which is generated in mice vaccinated with *Ag38*-transduced cells, and the partial or complete protection from intravenous challenge with parental cells which this protocol induces, *in vitro* and *in vivo* assays were performed to determine the antitumour activity of these antibodies.

5.2.1 *In vitro* complement assay

Complement-dependent cytotoxicity was tested by incubating sera at different dilutions with ^{51}Cr-labeled melanoma cells. As reported in figure 5.5, which shows the result of a representative experiment, the assay demonstrated the presence in these sera of antibodies able to recognise and lyse melanoma cells. Therefore, under the conditions of these *in vitro* experiments, complement fixation appears to be a plausible mechanism by which antibodies directed against melanoma cells might mediate tumour rejection.

5.2.2 *In vivo* passive immunization

To evaluate whether a protective immunity could be induced *in vivo* by the anti-melanoma specific antibodies, a passive immunisation experiment was performed using a pool of immunoreactive sera derived from protected *Ag38*-transduced melanoma immunised mice. Eight mice were challenged i.v. with $1x10^5$ parental melanoma cells and four mice were treated intraperitoneally with a pool of reactive sera at days 0 and + 2. The other four mice were treated, as a control, with an unrelated hyperimmune serum. Four weeks after the challenge, the number of experimental lung metastases was evaluated. As reported in Fig. 5.6, comparing to the control group a decrease in the number of lung metastases was observed (54 % of inhibition, $p>0.05$).

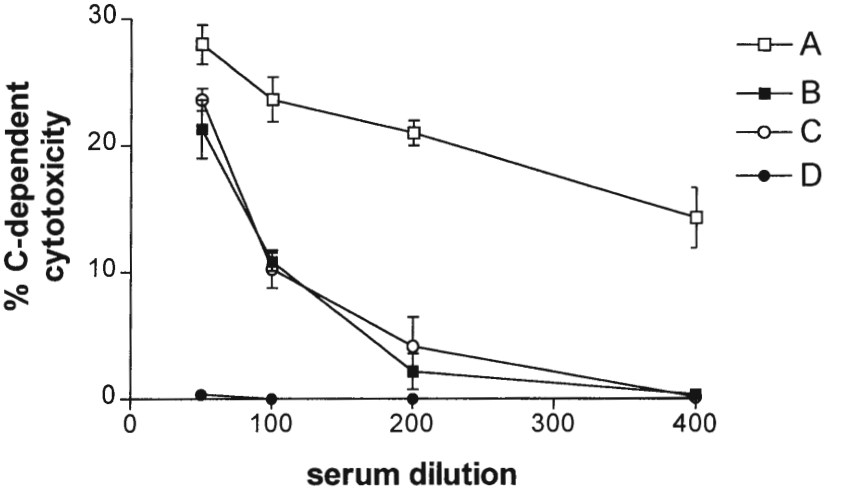


Fig. 5.5: Complement-dependent cytotoxicity of sera from protected mice

Complement-dependent cytotoxicity against B16 melanoma target cells of sera from vaccinated and protected mice (A, B, C) and of control serum (D). Values are expressed as means ± SD and refer to a representative experiment performed in triplicate.

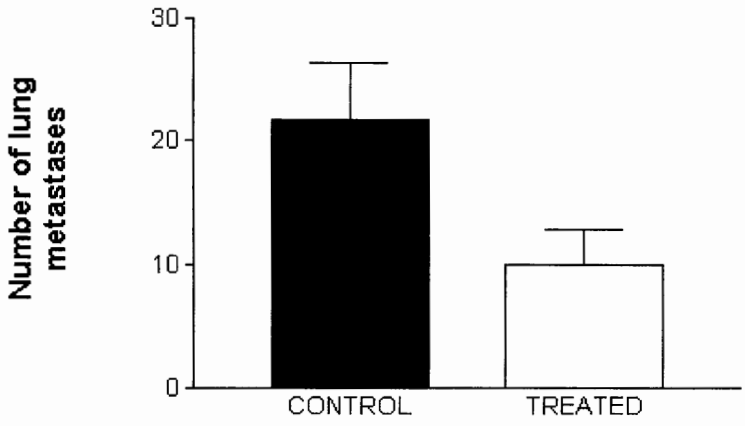


Fig. 5.6: In vivo passive immunisation with sera from protected mice

Mean number of lung metastases in mice i.v. challenged with B16 melanoma cells and intraperitoneally treated with a pool of reactive sera (TREATED) or with an unrelated control serum (CONTROL). P value > 0.05 by Unpaired t test.

However, due to the low number of mice, this decrease was below the level of significance. These data, although obtained in a few numbers of mice, support the possible role of antibodies in inhibiting *in vivo* growth of the melanoma tumour.

5.3 INITIAL APPROACHES TO IDENTIFY THE ANTIGENS RECOGNISED BY THE HUMORAL RESPONSE

Flow cytometry analyses were performed with immunoreactive sera, obtained from mice vaccinated with *Ag38*-transduced melanoma cells and protected from tumour challenge, and these revealed a strong reactivity against antigens expressed on the melanoma cell surface.

In order to identify the target molecules recognised by this humoral immune response, melanoma cells were cell-surface radiolabelled, solubilised, subjected to pre-clearing and immunoprecipitated with sera derived from protected mice. Subsequent separation by SDS-gel electrophoresis revealed a single, and specifically immunoprecipitated, band with a molecular weight between 70-85 kDa (fig. 5.7).

As a first approach to identify the antigen expressed on the melanoma cell surface which was recognised by sera from protected mice, an attempt was made to sequence the N-terminal residues of the immunoprecipitated molecule (this work was performed in collaboration with the Institute of Veterinary and Biochemical Physiology, University of Milan). To this aim, proteins immunoprecipitated with reactive sera were electroblotted onto a polyvinylidene difluoride membrane (PVDF), which represents a solid phase support for protein sequencing, and stained with Coomassie Blue to reveal the specific band. The bands, cut from the PVDF membrane, were then subjected to automated NH3-terminal Edman degradation.

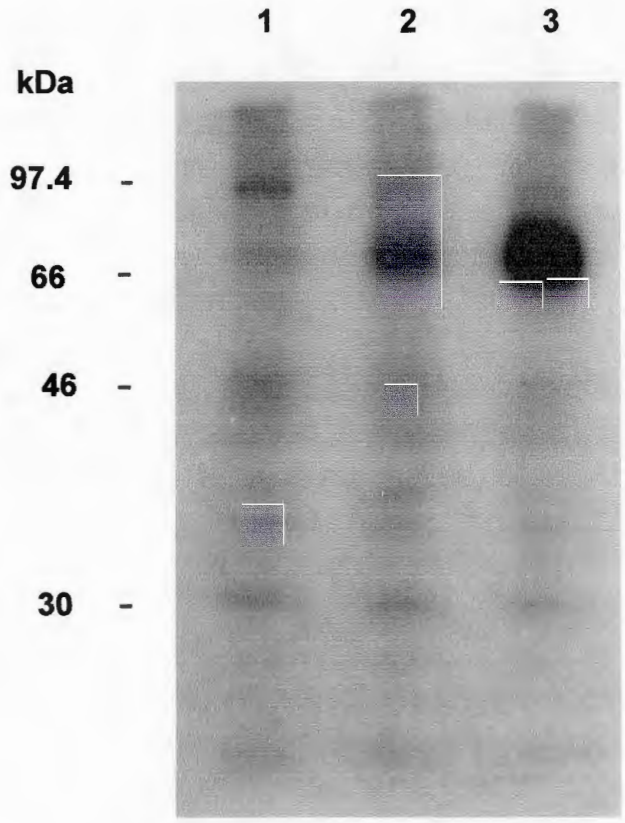


Fig. 5.7: Immunoprecipitation of soluble B16 cells with sera from protected mice

Immunoprecipitation of solubilised membrane-^{125}I-labeled B16 melanoma cells with a control serum (lane 1) or with sera collected from two survived B16-transduced immunised animals (lane 2 and lane 3).

Unfortunately, no information about aminoacid sequence was obtained by this approach, since the N-terminal ends of the bands appeared to be inaccessible to automated degradation.

As a second approach, the possible correspondence of the specific band immunoprecipitated from B16 cell surface with known murine melanoma antigens reported to have a similar molecular weight (between 70 and 85 kDa) was investigated. In particular, the following antigens, known to be expressed on murine B16 cells and to represent potential targets for an immune response, were evaluated:

i) the tyrosinase family, which includes tyrosinase (Jimenez et al., 1991), tyrosinase-related protein 1 (TRP-1) (Hara et al., 1995) and tyrosinase-related protein 2 (TRP-2) (Bloom et al., 1997),

ii) the gp100 protein (Schreurs et al., 1997),

iii) products encoded by endogenous retroviruses, known to be expressed on B16 melanoma cells (Li et al., 1998; Leong et al., 1988).

Since it was not known whether all these antigens, corresponding to melanosomal proteins or endogenous retroviral products, were expressed also on B16 cellular membranes, a preliminary analysis was performed by flow cytometry. Figure 5.8 shows the result of this analysis performed on B16 cells using the following specific antibodies:

- the AZN-LAM antiserum (kindly provided by Dr. M. W. J. Schreurs, University Hospital Nijmegen St Radboud, EX Nijmegen, The Netherlands) directed to gp100 protein,
- the αPEP antiserum (kindly provided by Dr. V Hearing, National Institutes of Health, Bethesda, MD) directed to TRP-2 antigen,
- the anti gp70 antiserum (kindly provided by Dr. M. L. Sensi, Istituto Tumori Milano, Italy) directed against MuLV gp70 protein,

- the TA99 monoclonal antibody (kindly provided by Dr. L. J. Old. Sloan-Kettering Cancer Center, New York, NY) directed against TRP-1,
- the T311 monoclonal antibody (kindly provided by Dr. L. J. Old, Sloan-Kettering Cancer Center, New York, NY) directed against tyrosinase,

As shown in the figure, TRP-1, TRP-2, gp100 and gp70 proteins were expressed at an high level on B16 cell surface, while a very low reactivity against the tyrosinase was observed using the specific antibody.

Precipitation of TRP-1, TRP-2, gp100 and gp70 antigens from solubilised cell-surface radiolabelled B16 cells was performed therefore using the specific antibodies.

Figure 5.9 shows the result of the experiments which compares, in the same run-conditions, the products precipitated by AZN-LAM antiserum (a, lane 2), αPEP antiserum (a, lane 3), anti gp70 antiserum (a, lane 4), TA99 monoclonal antibody (b, lane 1) and immunoreactive serum from protected mice (a, lane 5 and b, lane 3).

As illustrated in the figure 5.9, the immunoreactive serum precipitated from B16 cell surface proteins a molecule with a weight higher than that immunoprecipitated by anti-TRP-1 and anti-TRP-2 sera, and similar to that immunoprecipitated by an antiserum directed against murine leukaemia virus (MuLV) gp 70 product. No precipitation was observed using anti-serum against gp100.

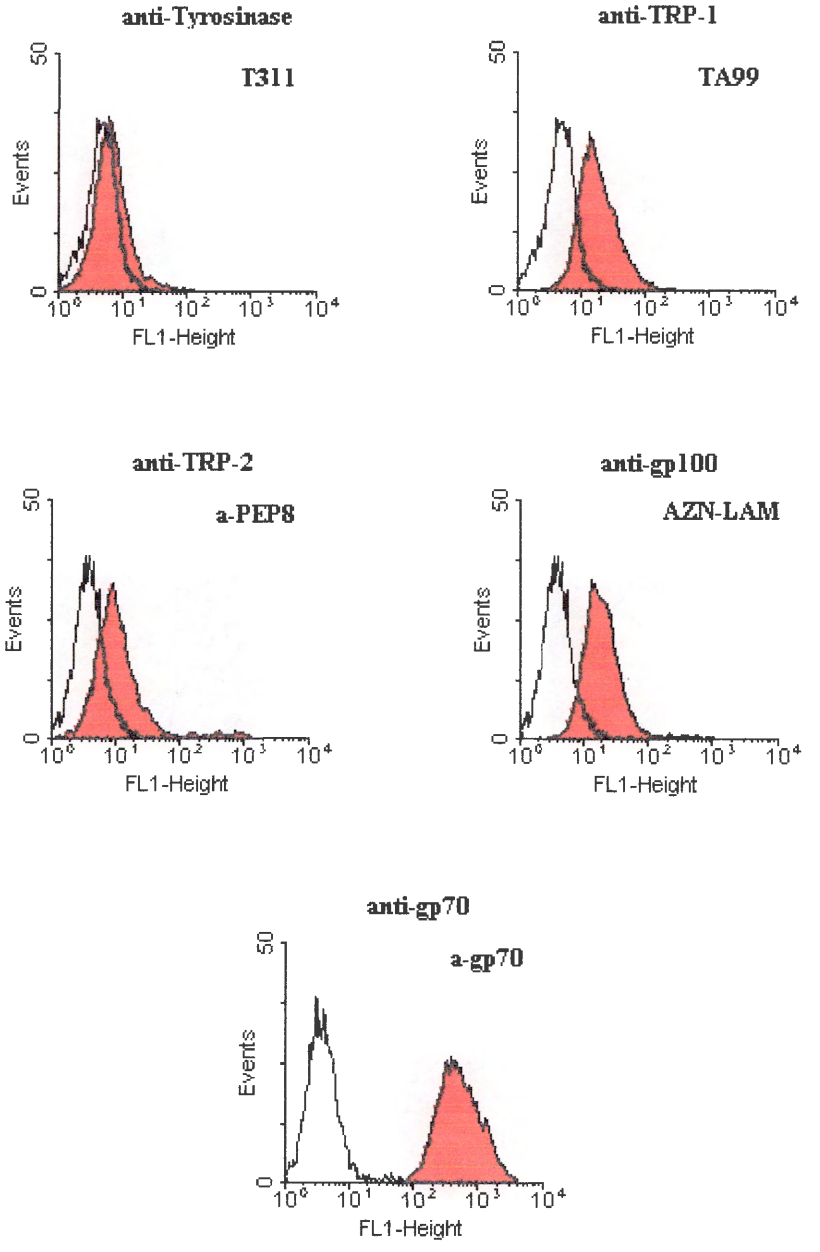


Fig. 5.8: FACScan analysis of melanoma antigens expression on B16 cell surface.

FACScan analysis was performed on B16 cells incubated with T311 antibody, or TA99 antibody, or aPEP8 antiserum, or AZN-LAM antiserum or agp70 antiserum. Cells were stained by an indirect method using FITC anti-mouse IgG+IgM (T311 and TA99), or FITC anti-rabbit IgG+IgM (aPEP8 and AZN-LAM) or FITC anti-goat IgG+IgM (αgp70). Open areas indicate cells stained with secondary antibody alone.

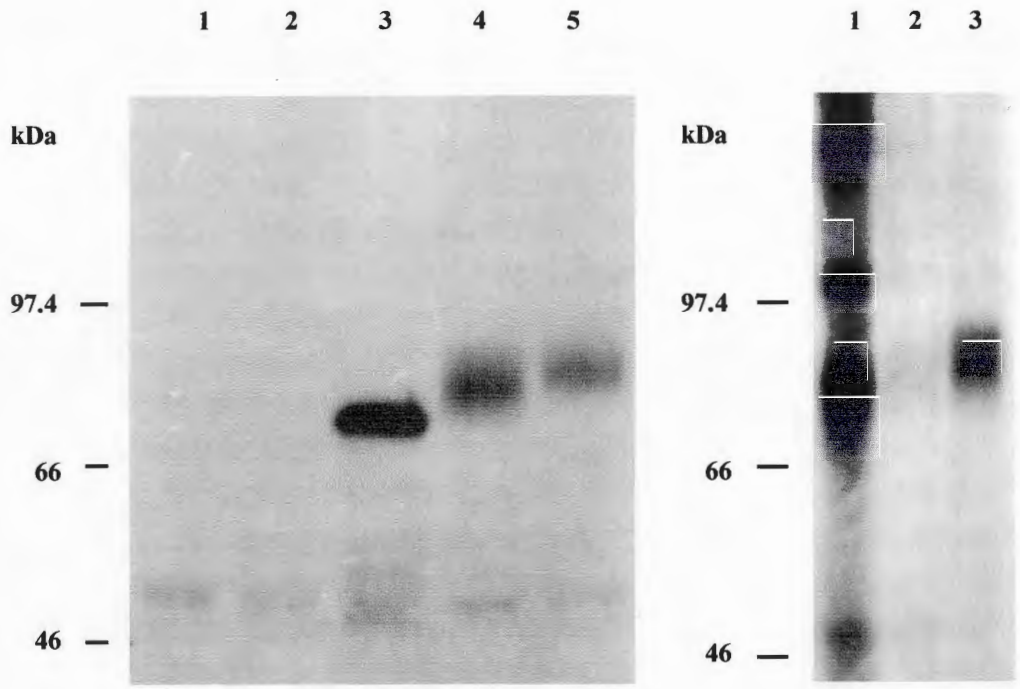


Fig. 5.9: Comparison of products immunoprecipitated from solubilised B16 cells with reactive sera and with anti-melanoma antigens antibodies

Immunoprecipitation of solubilised membrane-^{125}I-labelled B16 melanoma cells with:

a) control serum (lane 1), AZN-LAM serum (anti-gp100) (lane 2), aPEP8 serum (anti-TRP-2) (lane 3), agp70 serum (anti-viral gp70 env) (lane 4), serum from a survived *Ag38*-transduced immunised animal (lane 5);

b) TA99 mAb (anti-TRP-1) (lane 1), control serum (lane 2), serum from a survived *Ag38*-transduced immunised animal (lane 3).

5.4 DEFINITIVE IDENTIFICATION OF ANTI-RETROVIRAL HUMORAL RESPONSE

In view of the result obtained using the strategy describe above, it was of interest to perform further experiments to evaluate the possible correspondence of the molecule immunoprecipitated by the reactive serum with an endogenous retroviral product.

5.4.1 Reactivity against PT cells

The murine NIH/3T3-transformed pT cell line and its variant pT-N, derived after transplantation *in vivo* into nude mice, were used to perform a FACScan analysis with the reactive serum. As a consequence of one passage into nude mice, known to harbor endogenous xenotropic and ecotropic C-type virus, the pT-N variant acquired murine type C viruses and therefore expresses retroviral products.

As shown in Figure 5.10, flow cytometry analysis performed on the two tumour cell variants revealed a strong reactivity of the immunoreactive sera only on the viruses-infected line. This result therefore is consistent with the hypothesis of the presence, in reactive serum, of antibodies directed against endogenous retroviral antigens.

5.4.2 Immunoprecipitation of retroviral products

Conclusive results were obtained by performing immunodepletion and immunoprecipitation experiments from solubilised cell-surface radiolabelled B16 melanoma cells.

In a first experiment, an antiserum directed against total murine leukaemia virus products was used to remove viral antigens from solubilised cell-surface radiolabelled B16 melanoma cells.

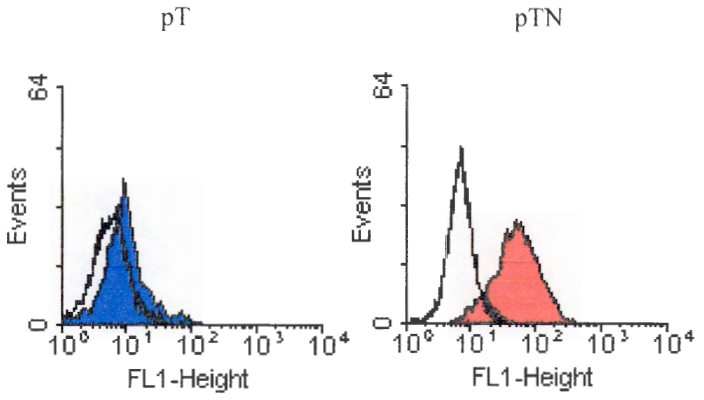


Fig. 5.10: FACScan analysis of sera reactivity on pT and pT-N tumour cell lines

FACS analysis of pT and pT-N tumour cells incubated with immunoreactive serum diluted 1:100 and stained with goat FITC-anti-mouse IgG+IgM. Open areas indicate cells stained with secondary antibody alone.

Complete immunodepletion of virus products was obtained by three cycles of subsequent immunoprecipitations with the antiserum (lane 1, 2 and 3).

As shown in Fig 5.11, after immunodepletion of virus products, precipitation was no longer observed with the sera from protected mice (lane 4).

To better define the viral molecule recognised by reactive serum, further experiments were performed using an antiserum directed against the virus envelope protein gp70, able to recognise both the virus envelope mature protein and its polyprotein precursor of 85 kDa (Traversari et al., 1989). In this experiment, the radiolabelled cell-surface B16 melanoma cells were subsequently immunoprecipitated three times with immunoreactive murine serum to remove the target molecule specifically recognised by this serum. The depleted supernatant was then subjected to immunoprecipitation with anti-gp70 serum. As shown in Figure 5.12, in this condition, only a narrow band with a molecular weight of 70 kDa was still immunoprecipitated. Since the anti-gp70 serum was able to precipitate from the non-depleted supernatant both the virus envelope protein gp70 and its polyprotein precursor of 85 kDa (not completely resolved in the condition of gel running), this result demonstrated that the immunoreactive sera specifically recognised the 85-kDa polyprotein precursor of the gp70 virus envelope protein.

In summary, in mice immunised with *Ag38*-transduced melanoma cells and protected from tumour challenge a strong humoral response directed against endogenous retroviral antigens was detected. These antibodies had the ability to immunoprecipitate their target molecules, expressed on the B16 cell surface, from solubilised cells.

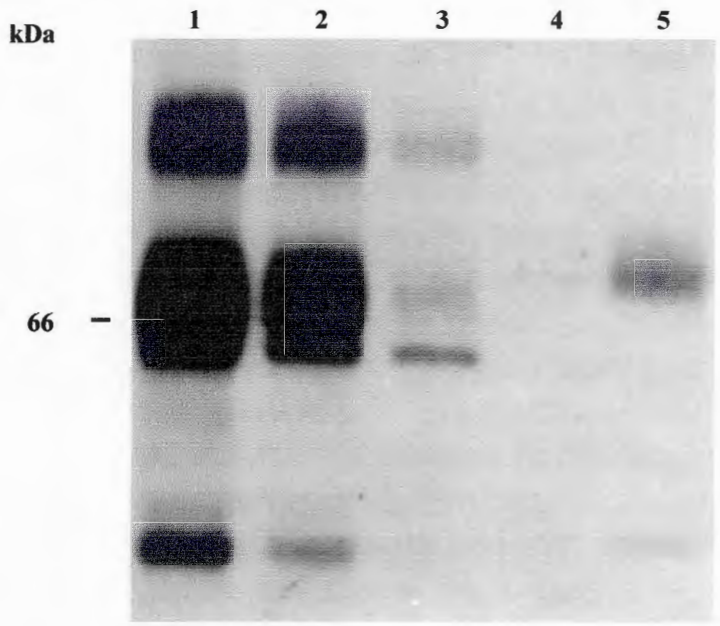


Fig. 5.11: Immunoprecipitation with reactive sera after depletion with an anti-total virus serum

Immunoprecipitation of solubilised ^{125}I-labeled B16 melanoma cells with a representative serum sample from a protected mouse before (lane 5) or after (lane 4) immunodepletion performed by sequential immunoprecipitation of solubilised extracts three times with anti-total virus (murine leukemia virus) serum. Proteins conjugated to Sepharose in each subsequent passage were recovered and loaded (lanes 1, 2 and 3, respectively). After three cycles of absorption, proteins remaining in the supernatant were subjected to immunoprecipitation with serum.

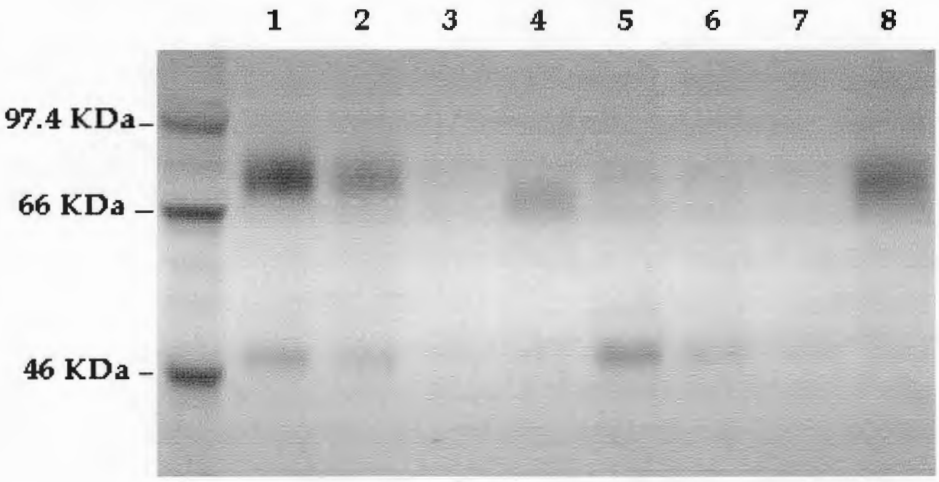


Fig. 5.12: Immunoprecipitation with anti-gp70 serum after immunodepletion with a reactive serum

Immunodepletion of ^{125}I-labeled and solubilised B16 melanoma cells, performed by sequential immunoprecipitations as described in Fig. 5.10, with serum from a protected transduced B16-immunized mouse (lanes 1, 2 and 3, for each subsequent immunoprecipitation), followed by immunoprecipitation with anti-gp70 serum (lane 4) and immunodepletion with a control serum (lanes 5, 6 and 7), followed by immunoprecipitation with anti-gp70 serum (lane 8).

5.5 EVALUATION OF THE HUMORAL RESPONSE AGAINST LINEAGE-SPECIFIC ANTIGENS

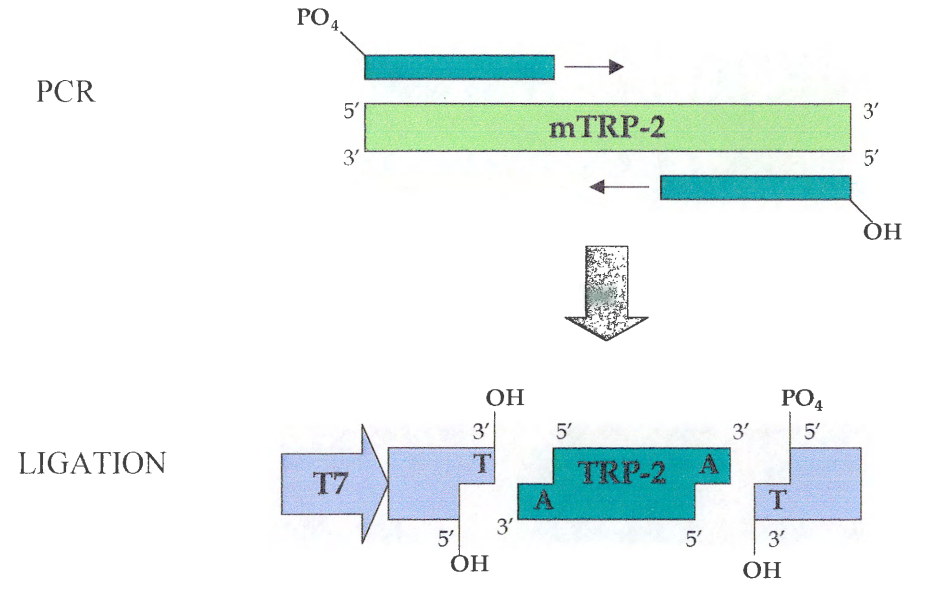
Even though the antibody response was directed mainly against endogenous retroviral antigens, immunisation with *Ag38*-transduced cells may also have induced a lesser response against tumour associated antigens. In addition, these antibodies may have not been able to immunoprecipitate their target molecules from solubilised B16 cells under the conditions of the above experiments. Therefore, further analyses were performed to investigate the presence of antibodies specifically directed to melanocyte lineage-specific antigens in sera of protected mice.

This study particularly focused on the evaluation of the presence of anti-TRP-2 antibodies, since this antigen has been shown to be the main target of an immune response against B16 cells. Indeed, it has been described that CTL clones recognising the TRP-2 antigen can be raised from B16 immunized-C57BL/6 splenocytes after repeated stimulation *in vitro* with IL-2 and B7.1 transfected B16 cells (Bloom et al., 1997).

5.5.1 Lack of a detectable humoral response against TRP-2

In order to evaluate the presence of antibodies directed against TRP-2 antigen in sera of protected mice, the murine *TRP-2* coding region was cloned by RT-PCR into the pCR3.1Uni eukaryotic vector and the protein was produced using an *in vitro* transcription-translation system.

Total RNA obtained from B16 cells was reverse-transcribed and the cDNA fragment corresponding to the open reading frame (ORF) of murine *TRP-2* was amplified by PCR using forward and reverse primers reported in Figure 5.13.



TRP-2	primer
sense (nt 405-434)	5′-ATGGGCCTTGTGGGATGGGGGCTTCTGCTG-3′
antisense (nt 1958-1929)	5′-CTAGGCTTCCTCCGTGTATCTCTTGCTGCT-3′

Fig. 5.13: Cloning of murine *TRP2*

Schematic diagram describing the cloning of murine *TRP-2* in pCR3.1Uni vector. The open reading frame of murine *TRP-2* was reverse transcribed and amplified by PCR with 3'-A overhangs at each end. PCR product was then directly ligated in the linearised vector which contained 3'-T overhangs at each end. Nucleotide position and sequences of sense and antisense primers used for amplification are reported in the text box.

As shown in the picture in the same figure, the reaction product was ligated directly into the vector to obtain the pCR3.1mTRP2 construct.

To verify the integrity of the construct, restriction analysis and automated DNA sequencing of the TRP2 insert were performed.

The *TRP-2* cDNA cloned into the pCR3.1Uni vector was in the correct orientation for expression of TRP-2 from the T7 promoter. The insert was *in vitro* transcribed and translated by a TnT T7-coupled reticulocyte lysate system to obtain the TRP-2 protein labelled with ^{35}S-methionine.

As shown in figure 5.14 A), analysis by SDS-PAGE and autoradiography of the purified labelled molecule revealed a single protein product with a molecular weight in agreement with that predicted from the amino acid sequence of the protein.

In order to evaluate the presence of antibodies directed against the TRP-2 antigen in sera from protected mice, the ability of sera to immunoprecipitate the labelled molecule was analysed.

As shown in figure 5.14 B), the level of radioactivity precipitated by sera obtained from three different protected mice was very low and similar to the background obtained by precipitation with an unrelated hyperimmune serum. However, in the same conditions, the ^{35}S-TRP-2 molecule was precipitable by the rabbit anti-PEP8 serum, which recognises a peptide consisting of the C-terminal 14 amino acids of murine TRP-2.

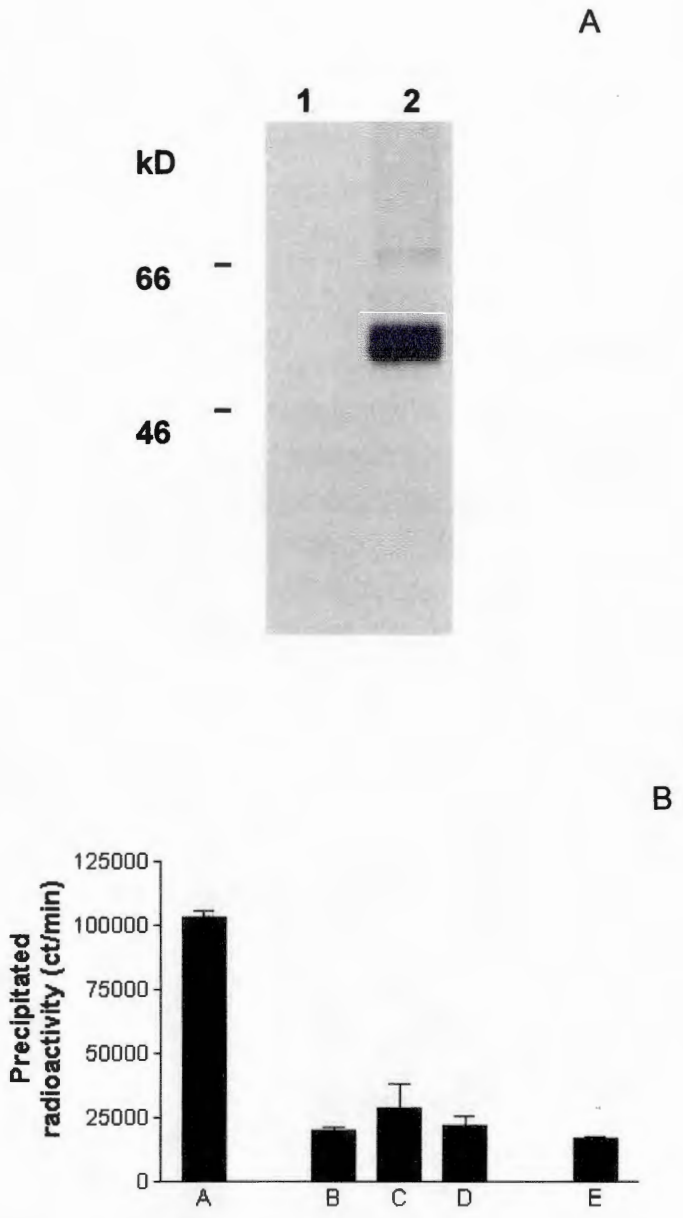


Fig. 5.14: Reactivity of sera against *in vitro* transcribed-translated TRP-2

A) SDS-PAGE and autoradiography of *in vitro* transcribed-translated ^{35}S-TRP-2 (lane 2) and control reaction with empty vector (lane 1).

B) Immunoprecipitation of ^{35}S-TRP-2 with (A) anti-PEP8 serum, (B, C, D) hyperimmune sera from three protected mice immunised with transduced B16 cells and (E) an unrelated hyperimmune mouse serum. Precipitated radioactivity is expressed as ct/min and represents the mean (± standard deviation) calculated from two independent experiments.

To exclude the possibility that the glycosylation-free *in vitro* transcribed-translated ^{35}S-TRP-2 used was not recognised because of the lack of some epitopes, the murine *TRP-2* cDNA was expressed in a non-melanoma cell line, which does not endogenously express this protein. The Chinese Hamster Ovary tumour cell line was chosen for this experiment.

CHO cells were transfected with the pCR3.1-mTRP2 construct by lipofection technology and stably transfected clones were selected in G418.

Northern blot analysis performed using a probe corresponding to the entire ORF of the *TRP-2* gene revealed the specific expression of TRP-2 transcript in stable transfected clones (Fig. 5.15 a).

Western analysis of total protein extracts obtained from *TRP-2*-transfected cells using the anti-murine TRP2 rabbit aPEP8 serum revealed the expression of TRP-2 protein in *TRP-2*-transfected CHO cells (Fig. 5.15 b).

The reactivity of sera from protected mice on TRP2-expressing CHO cells was evaluated by Western blot analysis and immunofluorescence analysis. Figure 5.15 c shows the result of Western blot analysis, performed on total protein extract from TRP2-transfected or mock cells with the reactive sera. No specific band, detectable only in TRP2-expressing cells, was revealed using this approach.

Immunofluorescence analysis performed on permeabilized cells also failed to reveal any difference in the reactivity of sera detected on mock and on *TRP-2*-transfected CHO cells (Fig.5.16).

In summary, no reactivity was found in sera from protected mice against TRP2 antigen, either obtained by *in vitro* transcription-translation or stably expressed in a non-melanoma cell line.

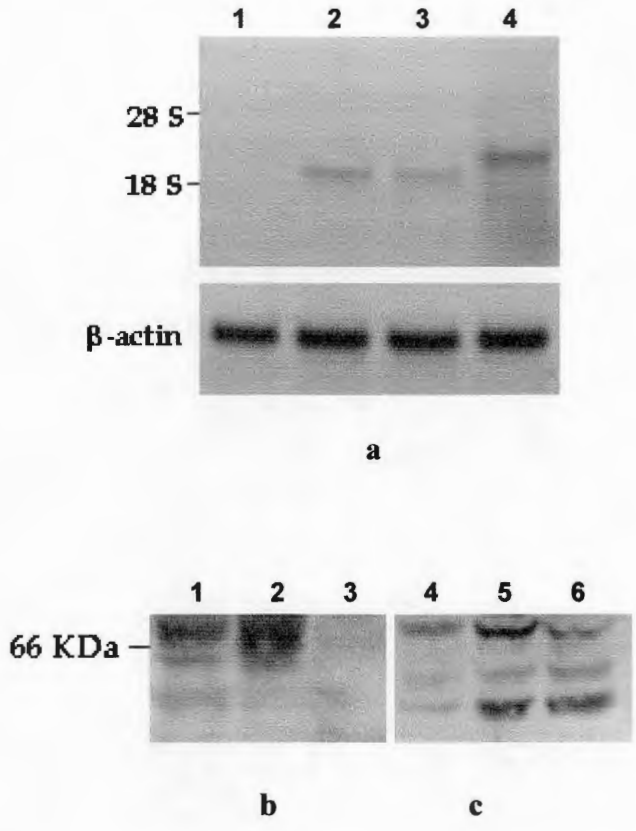


Fig. 5.15: *TRP-2* gene expression in CHO-transfected cells

a) Northern blot analysis of the expression of murine *TRP-2* mRNA in transfected CHO clones: 10 μg of total RNAs from mock cells (lane 1), from two representative clones of transfected CHO cells (lanes 2 and 3), and from B16 cells (lane 4) was loaded in each lane. Expression of the β-actin gene was included as control. The position of 28S and 18S RNAs is indicated.

b) Western blot analysis of the expression of murine TRP-2 in transfected CHO cells: 100 μg of total soluble extract from B16 cells (lane 1), from one representative clone of transfected cells (lane 2), and from mock cells (lane 3) was loaded in each lane and reacted with anti-PEP8 serum.

c) Western blot analysis to evaluate reactivity of sera from protected mice on murine TRP-2 transfected CHO cells: total soluble extract from B16 cells (lane 4), from one representative clone of transfected cells (lane 5), and from mock cells (lane 6) was loaded in each lane as in figure b) and reacted with serum diluted 1:500

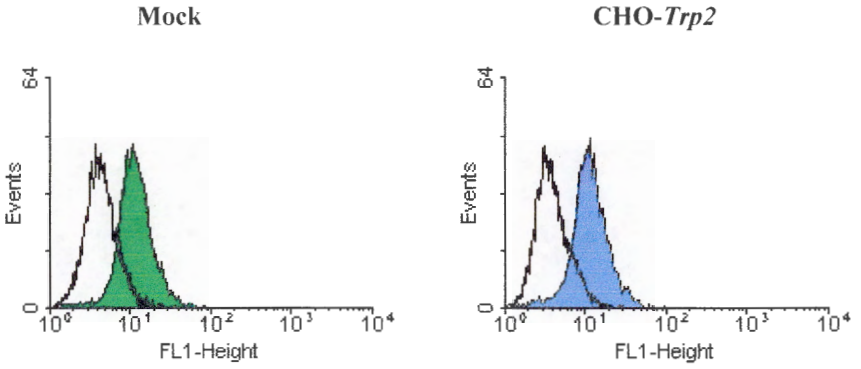


Fig. 5.16: FACScan analysis of sera reactivity on *TRP2*-transfected CHO cells

Immunofluorescence analysis on permeabilised mock and *TRP-2*-transfected CHO cells after incubation with reactive serum at 1:100 and staining with goat FITC-anti-mouse IgG+IgM. Open areas indicate cells stained with secondary antibody alone.

5.5.2 Lack of a detectable humoral response against tyrosinase, TRP-1 and gp100 antigens

The presence in sera of protected mice of antibodies directed to other melanocyte lineage-specific antigens was then analysed.

Tyrosinase and gp100 antigens were immunoprecipitated from total protein extract of B16 cells with the specific antibodies. Each product obtained from the immunoprecipitations was loaded in duplicate and separated by SDS-PAGE. Western blot analysis was then performed using the specific antibodies, to verify the presence of the protein antigens or, using reactive sera from protected mice, to evaluate the reactivity against these antigens.

As shown in figure 5.17, gp100 and tyrosinase antigens were detectable by Western blot analysis with their specific antibodies AZN-LAM and T311. However, no specific bands were detectable in analysis performed under the same conditions using sera from protected mice (a).

Since TA99 antibody was not observed to work in the experimental conditions of Western blot, a different approach was used to verify the precipitation of TRP-1 antigen. ^{125}I-radiolabelled solubilised B16 cells were immunoprecipitated with TA99 antibody and the product obtained from precipitation was loaded in duplicate and separated by SDS-PAGE. Autoradiography and Western blot analysis were used respectively to evaluate the precipitation of the specific protein and the reactivity of sera. As shown in figure 5.17 b), autoradiography revealed the precipitation of a single labelled protein with a molecular weight in agreement with that of the TRP-1 antigen. However, Western blot analysis using sera from protected mice did not reveal the presence of antibodies able to recognise this molecule.

Therefore, no reactivity in the conditions of Western blot analysis was found in sera from protected mice against gp100, tyrosinase and TRP-1 antigens.

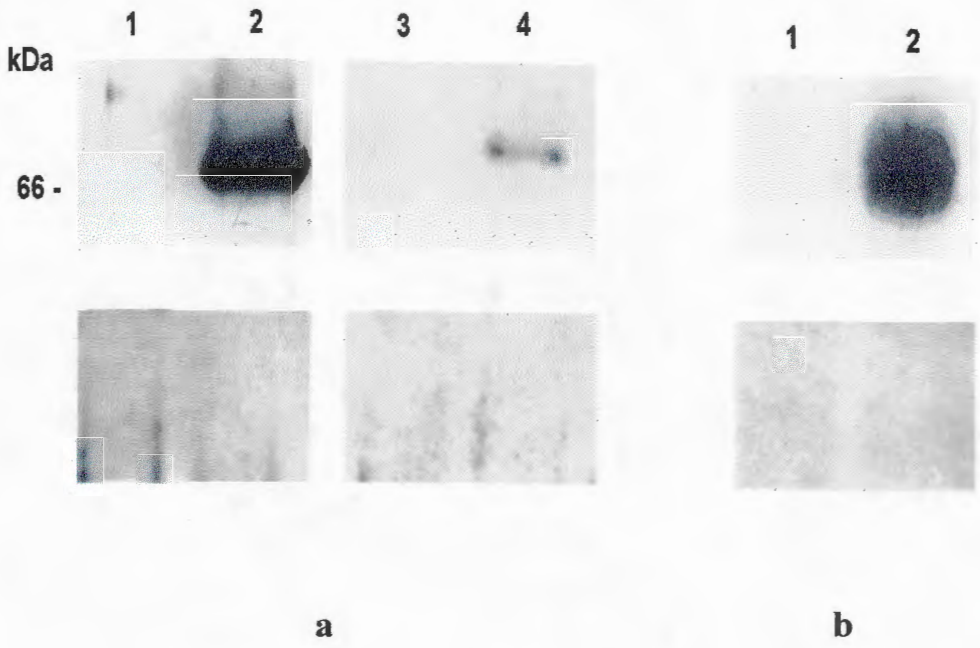


Fig. 5.17: Evaluation of sera reactivity against tyrosinase, gp100 and TRP-1 antigens

a) Immunoprecipitation of gp100 (lane 2) and tyrosinase (lane 4) from solubilised B16 cells and Western blot analysis with specific antibodies AZN-LAM and T311 (top) or with sera from protected mice (bottom). Immunoprecipitations with a normal rabbit serum (lane 1) and a normal mouse serum (lane 3) were loaded as negative control.
b) Immunoprecipitation of TRP-1 (lane 2) with TA99 antibody from ^{125}I-radiolabeled solubilised B16 cells: autoradiography of precipitated labeled TRP-1 (top) and Western blot analysis with sera from protected mice (bottom). Immunoprecipitation with a normal mouse serum (lane 1) was loaded as control.

5.6 CELLULAR RESPONSE

To evaluate whether vaccination with *Ag38*-transduced cells also induce the activation of a cellular response against melanoma tumour, a standard 4-h ^{51}Cr release assay was performed using fresh splenic lymphocytes obtained from mice vaccinated twice with a 4-week interval with 10^6 irradiated transduced-B16 cells and *in vivo* restimulated 5 days before the assay with *Ag38*-transduced cells.

No significant T cell cytotoxicity against melanoma cells (less than 4% of cytotoxicity at an effector:target ratio of 1:100) was detected in these conditions.

5.7 SUMMARY

Following vaccination with the *Ag38*-transduced cell vaccine a low serological response against melanoma cells was observed, while a high titre of antibodies directed against parental B16 cells, mainly of IgG2a isotype, were found in protected mice after challenge. These antibodies showed a complement-dependent cytotoxicity against melanoma cells *in vitro* whilst, *in vivo,* passive immunisation induced a decrease in the number of experimental B16 lung metastases.

Most of these antibodies were directed against endogenous murine leukaemia viruses, while no reactivity against melanocyte lineage-specific antigens was observed. In particular, no reactivity was found in sera from protected mice against TRP-2, either stably expressed in a non-melanoma cell line or obtained by *in vitro* transcription-translation, or against tyrosinase, TRP-1 and gp100 antigens immunoprecipitated from B16 cells.

No cytotoxic T cell activity against melanoma cells was detected in mice vaccinated with *Ag38*-transduced melanoma cells.

Thus, in the B16 murine model, the presence of dominant viral antigens induces a very strong humoral response that might be protective and may inhibit or mask the presence of minor clonotypes.

CHAPTER SIX

EVALUATION OF DIFFERENT VACCINATION PROTOCOLS AGAINST MELANOMA

6.1 EVALUATION OF DIFFERENT STRATEGIES BASED ON THE USE OF MYCOBACTERIAL PRODUCTS

Different vaccination strategies, based on the use of fractions or materials derived from *Mycobacterium tuberculosis*, were considered and compared to the use of an *Ag38*-expressing cellular vaccine. In particular, the administration of purified recombinant Ag38 protein, *Ag38* encoding DNA plasmid or Bacillus Calmette-Guérin (BCG) as vaccines to induce a protective immune response against melanoma were analysed.

6.1.1 Vaccination with recombinant Ag38 protein

The ability of recombinant Ag38 protein, administered in mice as a vaccine, to induce protection against tumour challenge of parental B16 cells was evaluated. To this aim, fourteen mice were vaccinated with 10 micrograms of purified Ag38 protein (Kindly provided by Dr. M. Singh, GBF, Braunschweig, Germany) administered subcutaneously in incomplete Freund's adjuvant (eight mice) or in the presence of 10^6 irradiated parental melanoma cells (six mice). The same treatment was repeated twice at 4-week intervals. Four weeks after the third vaccination, all mice were challenged intravenously with 5×10^5 viable parental cells.

The result of the experiment, represented as survival time, is reported in Figure 6.1. A low protection was observed in mice vaccinated with the protein administered in incomplete Freund's adjuvant or in the presence of irradiated parental cells.

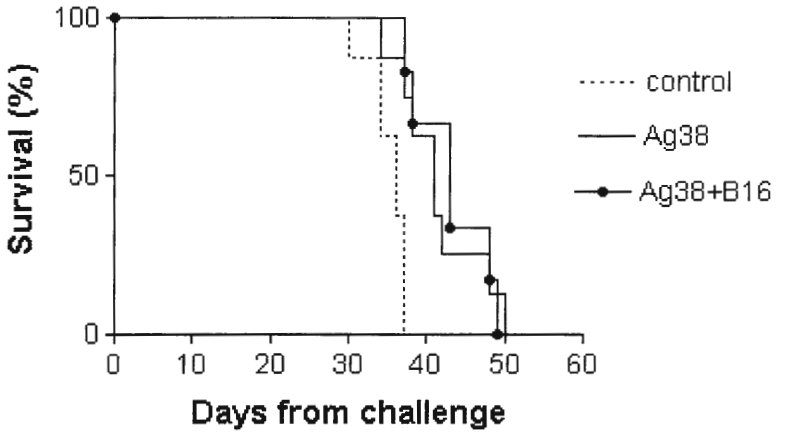


Fig. 6.1: Protection against experimental lung metastases by vaccination with Ag38 protein

Overall survival of mice either non-immunised (n = 8) (....) or immunised with purified Ag38 protein, administered in incomplete Freund's adjuvant (n = 8) (—) or in presence of irradiated melanoma cells (n = 6) (-•-). Mice were i.v. challenged with $5x10^5$ viable melanoma cells.

Thus, compared to vaccination with *Ag38*-expressing tumour cells, the level of protection against tumour evoked by the exogenous administration of the purified Ag38 protein, also in the presence of tumour cells, consistently was less effective.

6.1.2 Vaccination with *Ag38*-encoding DNA

In vivo vaccination with DNA plasmid encoding the Ag38 antigen as an alternative strategy to *in vitro* transduction was also evaluated.

To measure *Ag38* DNA vaccine efficacy, sixteen mice were injected intra-muscularly three times (at 3-week intervals) in both quadriceps with 60 micrograms, of an Ag38-encoding DNA construct and, one month after the third injection, intravenously challenged with 5×10^5 viable parental cells. A group of eight mice received only tumour challenge and was used as control.

As shown in Figure 6.2, a very low protection was observed after intramuscular immunisations with the Ag38 coding plasmid.

6.1.3 Vaccination with Bacillus Calmette-Guérin (BCG)

Application of Mycobacterium or its derivatives has a long history in tumour therapy approaches, including administration of BCG as a general immunostimulant or as an adjuvant administered together with tumour cells. The ability of whole live Bacillus Calmette-Guérin to stimulate an immune response able to protect against tumour challenge was therefore investigated.

Sixteen mice were injected subcutaneously with 10^6 live *M. bovis* BCG (Pasteur Mérieux Connaught, Italy) and one month later intravenously challenged with 5×10^5 viable parental cells. A group of eight mice received only tumour challenge and was used as control. As shown in Figure 6.3, as compared to control mice, immunisation with BCG generated a protective immunity, which resulted in a delay in tumour growth.

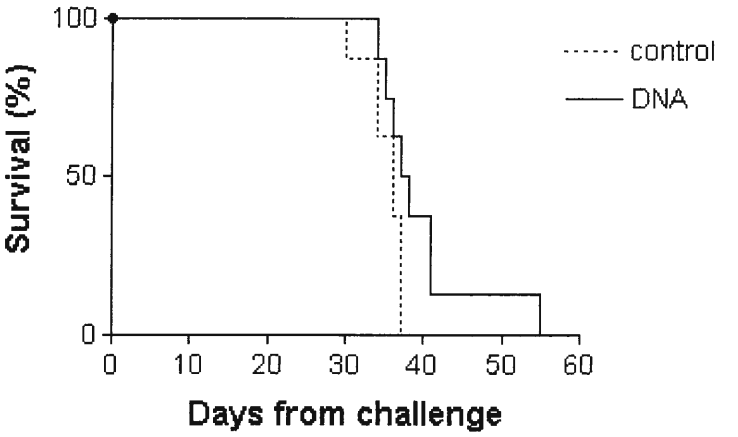


Fig. 6.2: Protection against experimental lung metastases by vaccination with *Ag38*-encoding DNA

Overall survival of non-immunised (n = 8) (...) or *Ag38* DNA-immunised (n = 8) (—) mice after i.v. challenge with $5x10^5$ viable melanoma cells.

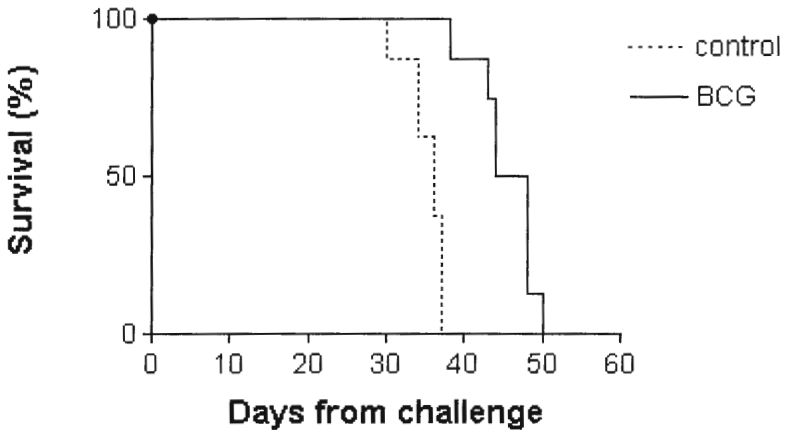


Fig. 6.3: Protection against experimental lung metastases by vaccination with BCG

Overall survival of non-immunised (n = 8) (...) or BCG-immunised (n = 8) (—) mice after i.v. challenge with $5x10^5$ viable melanoma cells.

However, this level of protection was less than that evoked by vaccination with *Ag38*-expressing tumour cells.

6.2 EVALUATION OF A RECALL WITH *AG38*-TRANSDUCED CELLS AFTER TUMOUR INOCULATION

To optimise the use of *Ag38*-transduced cells to enhance antitumour immune responses, studies were performed on vaccination schedules. In particular the effect of re-administration (recall) of irradiated transduced cells after tumour challenge was investigated.

A group of sixteen mice were vaccinated subcutaneously twice at a 4-week interval with 10^6 transduced irradiated melanoma cells and, four weeks after the second vaccination, challenged intravenously with 5×10^5 viable parental cells. Another group of eight mice received only tumour challenge and was used as control.

On days +1, +8 and +15 from tumour challenge eight of sixteen mice pre-vaccinated with transduced cells received s.c. a recall-treatment with 10^6 transduced, irradiated melanoma cells. The three groups of mice were evaluated for survival time.

As shown in Figure 6.4, in mice vaccinated with *Ag38*-transduced cells, the re-administration of transduced cells after tumour challenge resulted in a reduction of tumour protection induced by vaccination.

This result suggests that the recall treatment might stimulate a suppressive response able to abrogate the efficacy of vaccination.

In agreement with this hypothesis, it has been reported that vaccination with Mycobacterium proteins can induce an immunosuppressive response depending on dose and schedule by inducing secretion of immunosuppressive cytokines (Fine et al., 1990).

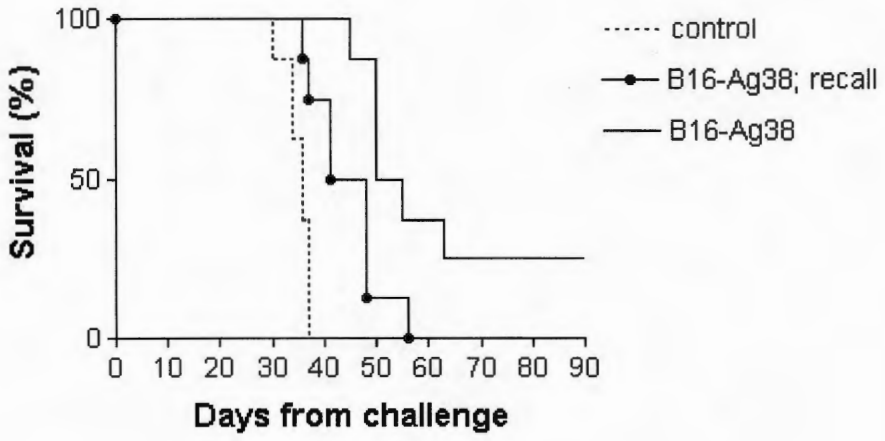


Fig. 6.4: Recall with *Ag38*-transduced cells after tumour inoculation

Evaluation of a recall with *Ag38*-transduced cells after tumour challenge with $5x10^5$ viable melanoma cells: overall survival of non-immunised mice (n = 8) (...), or of mice immunised with irradiated *Ag38*-transduced cells (n = 8) (—), or of mice immunised and re-called, after the tumour challenge, with irradiated *Ag38*-transduced cells (n = 8) (-•-).

6.3 EVALUATION OF A THERAPEUTIC PROTOCOL WITH *AG38*-TRANSDUCED CELLS

Experiments were performed to determine whether administration of *Ag38*-transduced cells might have a therapeutic effect on existing tumour.

Sixteen mice were intravenously challenged with 5×10^5 viable parental cells. On days +1, +8 and +15 from tumour challenge, eight of sixteen mice were subcutaneously injected with 10^6 *Ag38*-transduced irradiated melanoma cells. The remaining group of eight mice challenged with tumour was untreated and used as control.

As shown in Figure 6.5, no therapeutic effect was observed in mice with existing tumours since no differences in survival time between mice receiving only tumour challenge and mice treated with transduced cells after tumour challenge were detectable. However, as with other cancer vaccines, the limited efficacy in curing existing tumours is thought to be due mainly to the rapid growth of the tumour, leading to the death of mice before the activation of an efficient immune response. Furthermore, an inadequate penetration into the tumour site by immune cells and the escape from this immune response by tumour cells might represent additional limiting causes.

6.4 SUMMARY

Different vaccination strategies based on the use of mycobacterial products were compared to vaccination with *Ag38*-transduced tumour cells. Administration of purified Ag38 mycobacterial protein, DNA vaccination using the Ag38 encoding plasmid, or administration of BCG were evaluated. A very low protection was obtained using

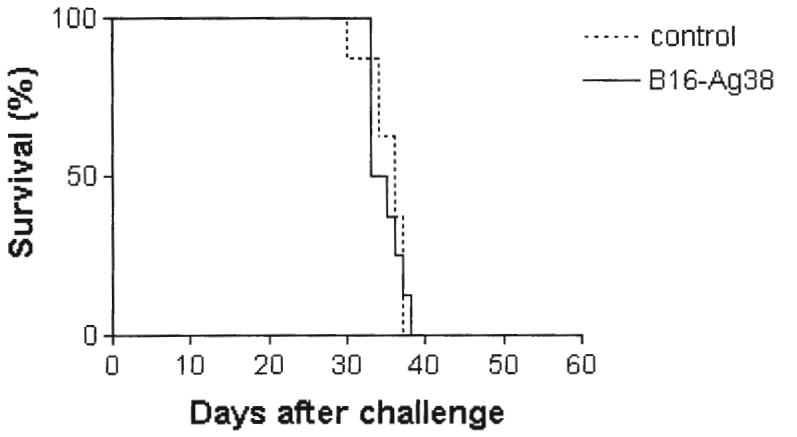


Fig. 6.5: Therapeutic protocol with *Ag38*-transduced cells

Evaluation of a therapeutic protocol with *Ag38*-transduced cells: overall survival of untreated mice (n = 8) (...), or of mice injected with irradiated *Ag38*-transduced melanoma cells (n = 8) (—) after i.v. challenged with $5x10^5$ viable melanoma cells.

immunisation schedules which employ the purified Ag38 protein or Ag38 coding plasmid; only a mild protection was observed by immunisation with BCG.

In experiments to determine whether mycobacterium-transduced cells have a therapeutic effect, no differences were observed between mice receiving only tumour challenge and mice treated with transduced cells after tumour challenge.

Moreover, in experiments to optimise the use of *Ag38*-expressing cells as tumour vaccine, the re-administration of transduced cells (recall) after tumour challenge unexpectedly resulted in a reduction of the tumour protection induced by vaccination.

CHAPTER SEVEN

MODULATION OF THE IMMUNE RESPONSE AGAINST SPONTANEOUS MAMMARY CARCINOMA BY VACCINATION WITH *Ag38*-TRANSDUCED CELLS IN FVB-NeuN MICE

7.1 THE FVB-NeuN TRANSGENIC MODEL

It was decided to evaluate the efficacy of vaccination with *Ag38*-transduced tumour cells in a model of spontaneous tumour development.

To this aim, an animal model that mimics the human breast carcinoma pathology was chosen. This model consists of mice transgenic for the rat *HER2/neu* protooncogene placed under the control of a specific promoter for mammary tissue, the mouse mammary tumour virus (MMTV) promoter.

In this strain, the transgene expression starts at about 2 months of age and at 4-5 months the mammary glands show hyperplasia and microfoci of in situ carcinomas. At about 6 months, female transgenic mice start to develop invasive carcinomas and by 12 months the tumour incidence reaches 100% (Guy et al., 1992; Boggio et al., 1998).

Transgenic mice, which expressed the transgene physiologically, were found to be rather tolerant to the transgene. Indeed, immunisation with transgenic tumour cells overexpressing HER2/neu did not induce immunity against tumour development.

7.2 PROTECTIVE IMMUNITY AGAINST SPONTANEOUS ARISING MAMMARY CARCINOMA BY VACCINATION WITH *AG38*-TRANSDUCED CELLS

Protection induced by vaccination with *Ag38*-transduced tumour cells against spontaneous tumour development was evaluated. The N202.1A tumour cell line, derived from a mammary carcinoma spontaneously grown in FVB-NeuN transgenic mice, was used to create the *Ag38*-expressing syngenic cell vaccine, as described above (see chapter 3.5).

In each experiment, proto-*neu*-transgenic virgin female mice were divided randomly into groups. The groups were always extremely homogeneous with respect to birth dates, thus avoiding the variability in the time of tumour appearance seen in our experiments, which may be associated to different seasonal development periods. In each experiment, vaccination was performed by two subcutaneous injections, at a 4-week interval, with 10^6 irradiated non-transduced tumour cells or 10^6 irradiated *Ag38*-transduced tumour cells. Mammary glands were inspected weekly and two perpendicular diameters of tumour masses were recorded. Differences between groups were analysed using the log rank test.

Figure 7.1 shows the result of a preliminary experiment performed using a small number of animals (5 for each group), which received the first vaccination treatment at the age of four months (i.e. 2 months before spontaneous tumour formation). All mice vaccinated with parental cells developed spontaneous tumours, whilst a partial protection was observed in the group of mice vaccinated with transduced cells. Indeed, in this group, a delay in tumour development in 1 out 5 mice and a complete protection at the end of the observation period (700 days) in 1 out of 5 mice was observed ($p = 0.366$).

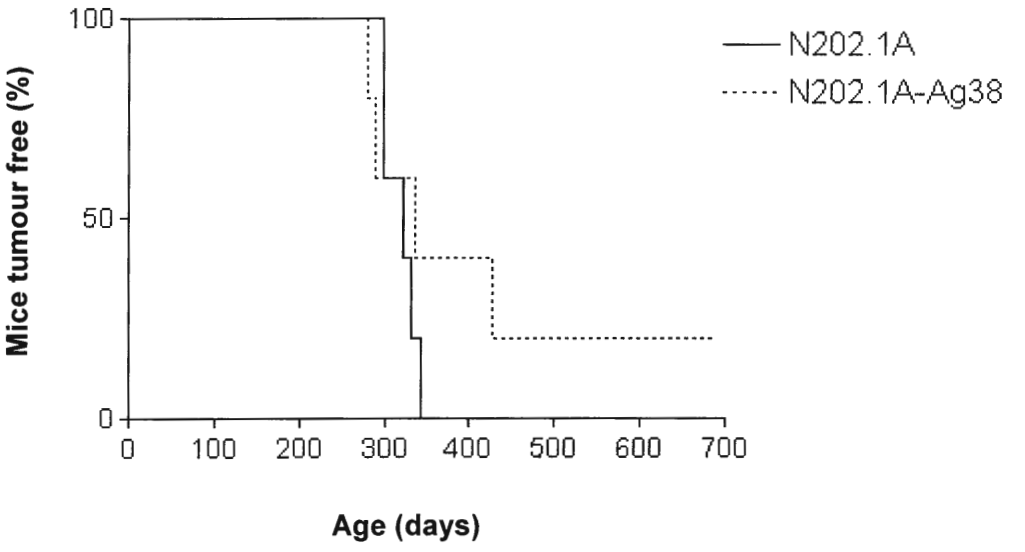


Fig. 7.1: Protection against spontaneous mammary tumour by vaccination with *Ag38*-transduced N202.1A cells in a preliminary experiment

Spontaneous mammary tumour development in proto-*neu* transgenic female mice vaccinated with non-transduced N202.1A cells (n = 5) and *Ag38*-transduced N202.1A cells (n = 5). P = 0.36, determined by log-rank test.

A second experiment was performed using a larger group of animals (11 for each group of vaccinations) and administering the first vaccination treatment at the age of two months. A third group of 8 mice was left untreated and used as a control to evaluate spontaneous tumour incidence.

The result of the experiment is represented in figure 7.2. In mice vaccinated with *Ag38*-transduced cells the onset of the first spontaneous mammary tumour was significantly delayed as compared with the control group ($p = 0.033$) (b), whereas no difference in tumour onset was observed between mice vaccinated with non-transduced N202.1A cells and controls ($p = 0.689$) (a). Moreover, the number of mammary glands with a palpable tumour was reduced in mice vaccinated with irradiated *Ag38*-transduced tumour cells as compared to mice vaccinated with non-transduced tumour cells: at 300 days, the mean number of tumours per mouse was 2.1 in this group as compared with 3.1 in mice vaccinated with non-transduced tumour cells.

These data indicate that the expression of Ag38 protein in tumour vaccine induces a protective immunity, which results in a delay in tumour appearance. Protection was achieved in this model, as it was in the melanoma model, against a tumour challenge, by using irradiated cells and after only two vaccinations.

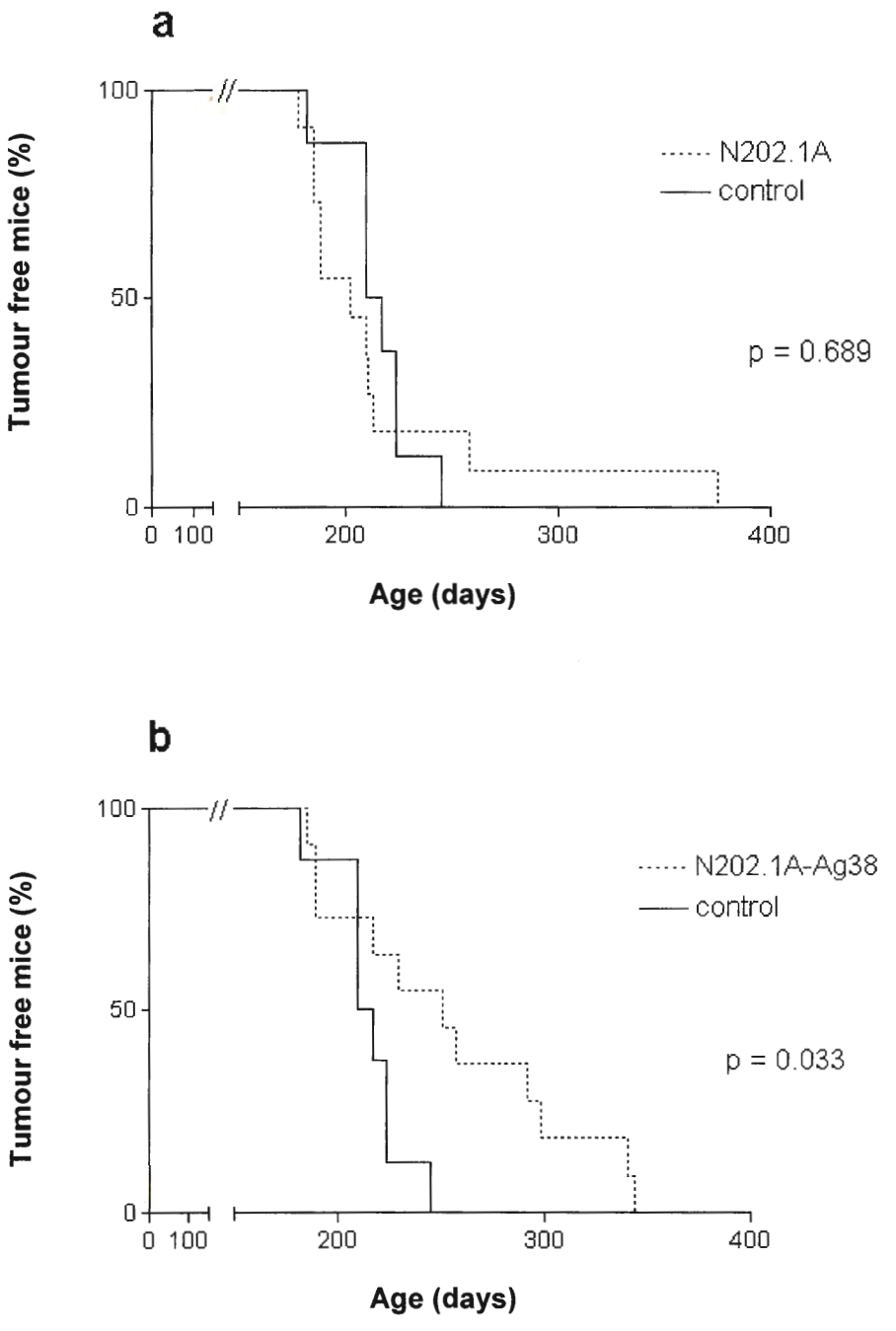


Fig. 7.2: Protection against spontaneous mammary tumour by vaccination with *Ag38*-transduced N202.1A cells

Spontaneous mammary tumour development in proto-*neu* transgenic female mice vaccinated with non-transduced N202.1A cells (n = 11) (**a**) and *Ag38*-transduced N202.1A cells (n = 11) (**b**). Control group n = 8. P values were determined by log-rank test.

7.3 SYNERGISTIC EFFECT OF IL-12

Bacterial DNA has been reported to induce macrophages to secrete IL-12 and, indeed, serum of mice maintained under specific pathogen-free conditions revealed a detectable levels of IL-12 after injection of bacterial DNA (Krieg et al., 1998). The protection observed with *Ag38*-transduced tumour cell vaccine could be related to a released of IL-12 induced by the presence of *M. tuberculosis* DNA in transduced cells. Therefore, it was investigated if a systemic co-administration of recombinant IL-12 (rIL12) together with the non-transduced tumour cells could induce the same anti-tumour response observed using transduced-tumour cell vaccine.

At the same time the anti-tumour immunity induced by *Ag38*-transduced cancer vaccine plus systemic co-administration of rIL-12 was evaluated. Indeed systemic administration of rIL-12 was recently reported to potentiate the effects of cancer vaccines engineered to secrete different cytokines (Vagliani et al., 1996; Taniguchi et al., 1998; Kurzawa et al., 1998).

Thirty six female transgenic mice were divided randomly into three groups (12 mice/group). Two groups were vaccinated at the age of 2 months with non-transduced or transduced cells as above, and systemically infused with rIL-12 immediately after vaccination with irradiated cells and on days +1, +2 and +3 (150 ng over 4 days). The third untreated group was used as control.

The result of the experiment is reported in figure 7.3. All mice vaccinated with non-transduced cells plus rIL-12 developed tumours with a slight delay in tumour onset as compared with the control group ($p=0.056$) (a), whereas spontaneous tumour development was significantly delayed in mice vaccinated with *Ag38*-transduced cells plus rIL-12, ($p=0.001$ versus control group) with 2 tumour-free mice at the end of the 400-day observation period (Fig. b).

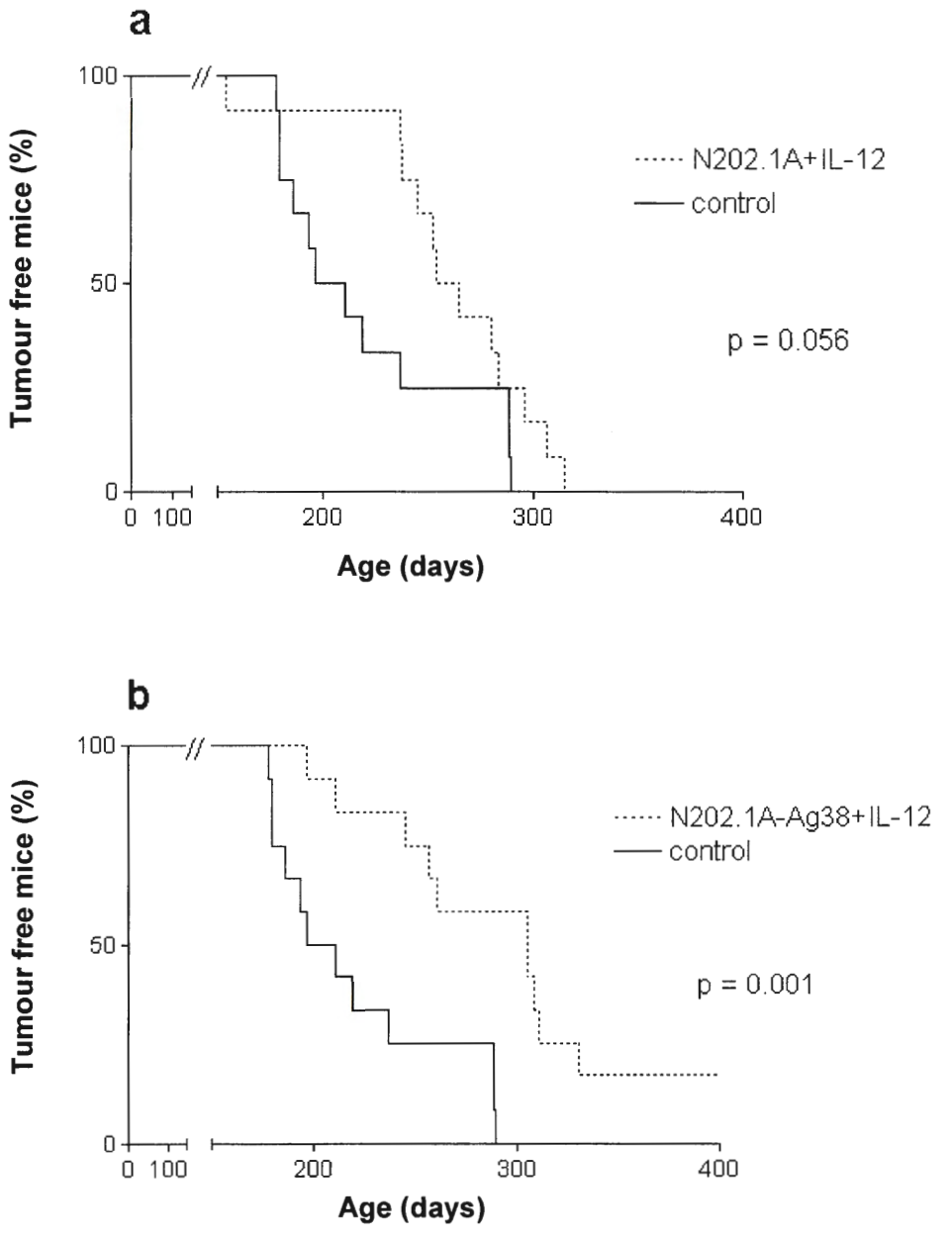


Fig. 7.3 Protection against spontaneous mammary tumour by vaccination with *Ag38*-transduced N202.1A cells plus IL-12

Spontaneous mammary tumour development in proto-*neu* transgenic female mice vaccinated with non-transduced N202.1A cells plus rIL-12 (n = 12) (**a**) and *Ag38*-transduced N202.1A cells plus IL-12 (n = 12) (**b**). Control group n = 12. P values were determined by log-rank test.

Moreover, the number of mammary glands with a palpable tumour was reduced in mice vaccinated with irradiated *Ag38*-transduced tumour cells: at 300 days, the mean number of tumours per mouse was 0.91 in this group as compared with 1.5 in mice vaccinated with non-transduced tumour cells plus rIL-12 and 2.08 in the control group.

Therefore, only a slight delay in tumour appearance was observed in mice systemically administered with non-transduced cells plus a low dose of recombinant IL-12 (rIL12). On the contrary, systemic co-administration of low dose rIL12 enhanced efficacy of *Ag38*-transduced cell vaccine, suggesting a synergy in the adjuvant potency of these reagents.

7.4 TH1- AND TH2-TYPE CYTOKINE SUBSETS INDUCED BY VACCINATION

The Th1- and Th2- responses, represented respectively by IFN-γ and IL-4 production, induced by the different vaccination treatments was evaluated.

Mice were vaccinated subcutaneously into the right hind footpad with 5×10^6 non-transduced or transduced irradiated cells alone or in the presence of rIL-12 injected intraperitoneally (150 ng/day on days 0, +1, +2, +3). Six days later, mice were sacrificed and popliteal lymph nodes were removed aseptically and pooled from 2 mice in each group. Lymphocytes were mechanically dissociated and cultured in plates precoated with 1 μg/well anti-CD3 MAb at 37°C for 18 h. Supernatant was then collected and tested for IFN-γ and IL-4 production.

Figure 7.4 shows the result of a representative experiment.

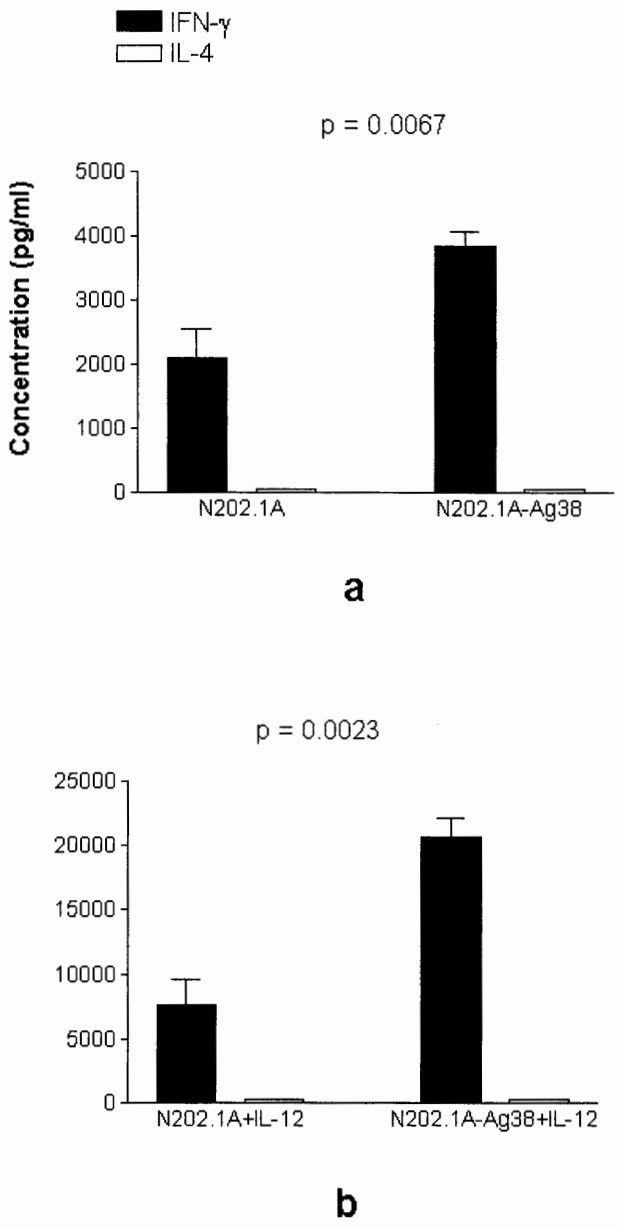


Fig. 7.4: Production of IFN-γ and IL-4 by popliteal lymph nodes of vaccinated mice

IFN-γ and IL-4 production by lymphocytes from popliteal lymph nodes of mice vaccinated with non-transduced N202.1A or *Ag38*-transduced N202.1A cells, without (a) or with rIL-12 (b), and stimulated with immobilised anti-CD3 mAb. Values were obtained by subtracting the cytokine levels in the supernatant of lymphocytes cultured in the absence of anti-CD3 antibody. P values were determined by two-tailed unpaired *t* test.

Lymphocytes obtained from popliteal lymph nodes of mice footpad-injected with *Ag38*-transduced N202.1A tumour cells alone or in the presence of rIL-12 produced significantly higher levels of IFN-γ, as compared respectively to lymphocytes obtained from mice vaccinated with non-transduced cells alone or in the presence of rIL-12. A greater than additive effect, between *Ag38*-transduced vaccine and rIL-12, was indicated by the greater level of IFN-γ secreted by draining lymph node cells from mice injected with the two reagents together. No production of IL-4 was observed in any group.

The induction of a preferential Th1 response by an endogenously expressed mycobacterial antigen is consistent with previous data obtained in the melanoma model.

7.5 ROLE OF IL-12 ON CYTOKINE PRODUCTION

Bacterial DNA has rapid immunostimulatory effects both *in vivo* and *in vitro*, mediated by the recognition of unmethylated CpG dinucleotides within specific flanking bases. CpG dinucleotides, referred to as CpG motifs, are under-represented and selectively methylated in vertebrate DNA, but are present at higher frequency and unmethylated in bacterial DNA. These motifs may have evolved as an ancestral mechanism used by the innate immune system to detect DNA of pathogens, such as bacteria and viruses (Hartmann et al., 2000). DNA containing these motifs has been reported to directly activate macrophages to secrete IL-12 (Halpern et al., 1996).

The role of IL-12 in mediating the vaccination efficacy of *Ag38*-tranduced cells was evaluated. The purpose of this study was to analyse whether the vaccination efficacy of *Ag38* transduction in tumour cells was mediated through the production of IL-12, as the analysis of *Ag38* gene sequence revealed the presence of 11 such CpG motifs, or is independent of IL-12. It was of interest to analyse this issue since it could demonstrate

if the cooperativity observed between Ag38 and IL-12 was due to an increased IL-12 production or due to additional factors related to *Ag38* expression that cooperate with IL-12 in inducing greater levels of IFN-γ.

It was, therefore, investigated if a neutralising antibody against IL-12 affected the dominant Th1 response to vaccination with *Ag38*-transduced cells. To this aim, mice were intraperitoneally treated for 4 days with 1 mg of purified rat anti-murine IL-12 p40 antibody (kindly provided by Dr. Trinchieri, The Wistar Institute of Anatomy and Biology, Philadelphia, PA), or, as control, with the same dose of an unrelated isotype antibody. Six hours after the first treatment with the antibody, mice were vaccinated s.c. into the right hind footpad with 5×10^6 transduced cells alone or with 5×10^6 non-transduced cells in the presence of rIL-12, injected i.p. (150 ng/day for 4 days). The experiment was then carried on as described above to evaluate IFN-γ production by draining lymph node cells.

Figure 7.5 shows the result of the experiment. In mice footpad-injected with *Ag38*-transduced cells, the administration of an anti-IL-12 antibody did not significantly change IFN-γ-production, as compared to control group (17% inhibition, p=0.603). On the contrary, in mice footpad injected with non-transduced cells and systemically infused with rIL-12, the antibody administration induced, as expected, a significant reduction in IFN-γ production (87% inhibition, p=0.0013). Therefore, IFN-γ release induced by vaccination with *Ag38*-transduced cells does not seem to be mainly mediated through the production of IL-12. This result suggests that the cooperativity observed on vaccination between Ag38 and IL-12 is probably attributable to additional Ag38-induced factors that cooperate with IL-12 in inducing greater levels of IFN-γ.

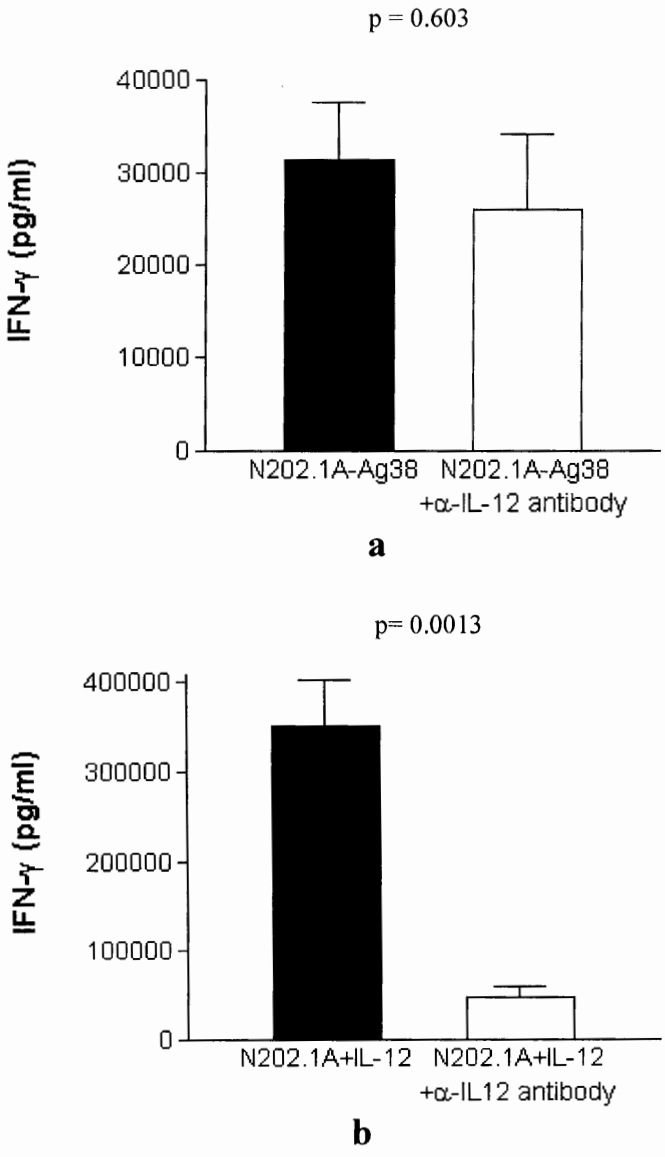


Fig. 7.5: Production of IFN-γ in response to vaccination in mice treated with anti-IL-12 antibody

IFN-γ production by lymphocytes from popliteal lymph nodes of mice i.p. treated with anti-murine IL-12 p40 antibody and vaccinated with *Ag38*-transduced N202.1A cells (**a**) or with N202.1A cells plus rIL-12 (**b**). Control mice not treated with the anti-IL-12 antibody received an i.p. injection with an unrelated antibody. Values were obtained by subtracting the cytokine levels in the supernatant of lymphocytes cultured in the absence of anti-CD3 antibody. P values were determined by two-tailed unpaired *t* test.

7.6 LYMPHOCYTE SUBSETS ACTIVATED BY VACCINATION

The type of T cell subset activated by vaccination and responsive to the tumour was then investigated.

To this aim, spleen cells of mice vaccinated with 10^6 irradiated *Ag38*-transduced tumour cells, or 10^6 irradiated non-transduced cells plus rIL-12 (150 ng/day on day 0, +1, +2, +3) or 10^6 irradiated non-transduced cells alone, were removed 7 days after vaccination and cultured *in vitro* with weekly restimulation with irradiated N202.1A tumour cells and low-dose IL-2 (25 U/ml). Syngeneic irradiated splenocytes were added to each well as feeder cells.

Only spleen cells of mice vaccinated with transduced tumour cells were able to survive to prolonged culture *in vitro* and to proliferate in the presence of tumour cells. After four *in vitro* stimulations, the presence of activated CD4+ or CD8+ lymphocytes and their cytokine production were determined by FACScan analysis of cells triple-stained with Cy-Chrome-labelled anti-CD8 or anti-CD4, FITC-anti-IFN-γ and PE-anti-IL-4 mAbs. As shown in figure 7.6, these cells were shown to be mainly $CD3^+$ $CD8^+$ and to produce IFN-γ.

7.7 RECOGNITION OF MAMMARY CARCINOMA CELL LINES BY ACTIVATED LYMPHOCYTES

The production of IFN-γ in response to the specific recognition of tumour antigens was then evaluated.

In a first experiment, the recognition of N202.1A parental cells was investigated. $CD3^+$ $CD8^+$ lymphocyte population obtained from mice immunised with *Ag38*-transduced

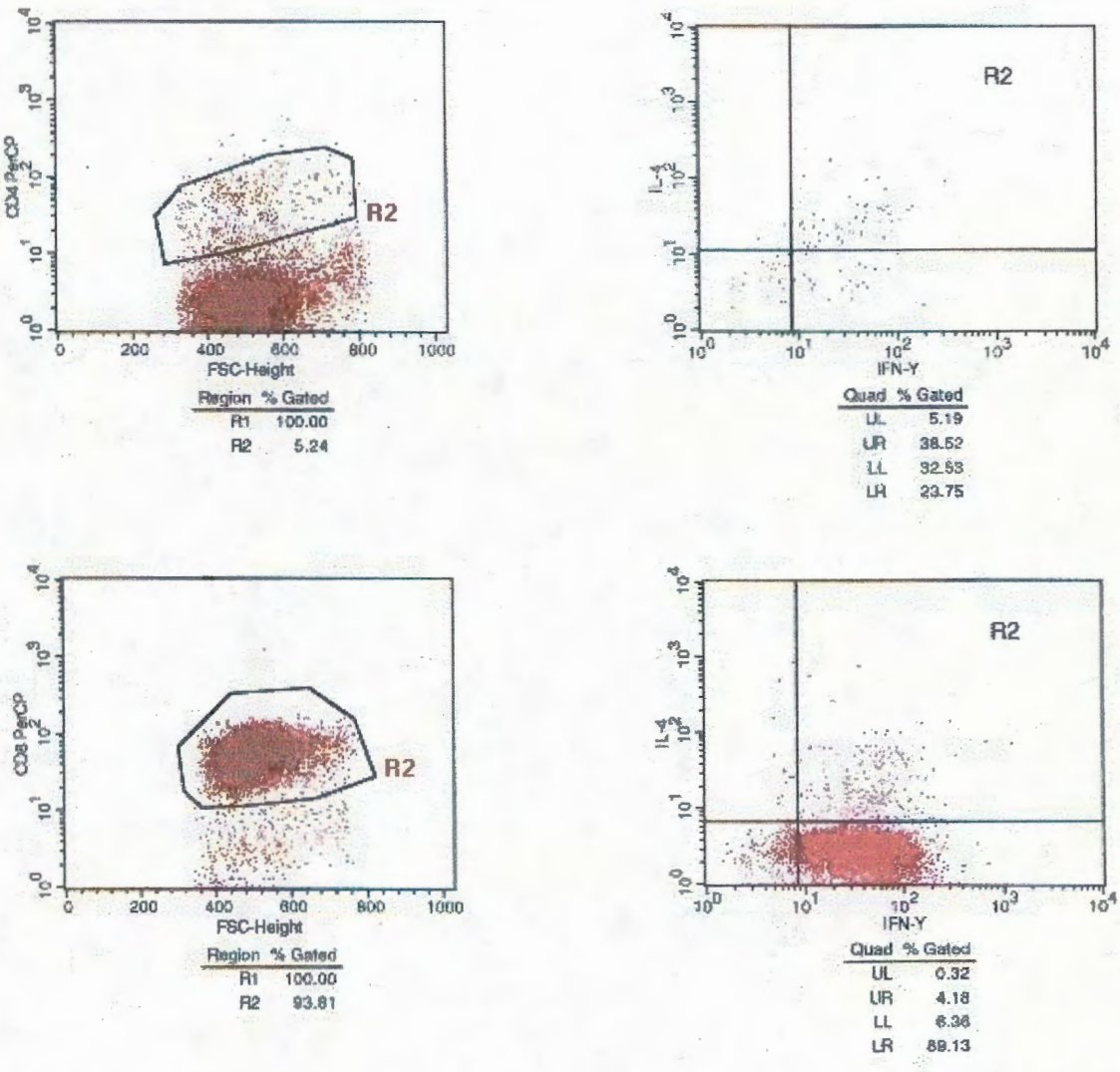


Fig. 7.6: Analysis of lymphocytes subset(s) activated by vaccination

Characterisation of splenocytes obtained from mice vaccinated with *Ag38*-transduced N202.1A cells and restimulated *in vitro* with N202.1A tumor cells and IL-2. Flow cytometry analysis of cells stimulated with immobilised anti-CD3 mAb, in the presence of monensin, and triple-stained with Cy-Chrome-anti-CD8 or Cy-Chrome anti-CD-4, PE-anti-IL-4 and FITC-anti-IFN-γ mAbs. Analysis of cytokine production was performed setting a gate on CD4- (top) or CD8- (bottom) positive cells.

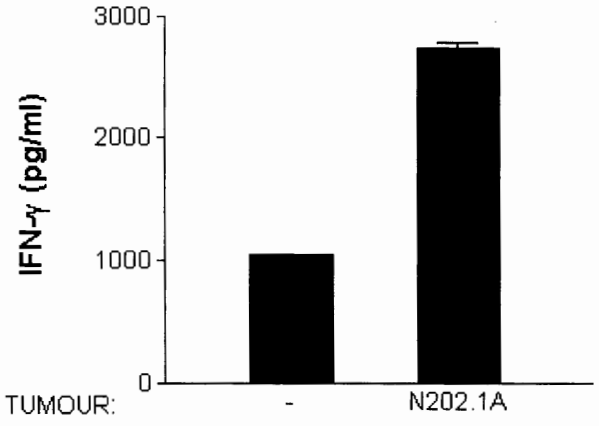
cells was *in vitro* stimulated with irradiated N202.1A cells, or cultured alone, in the presence of IL-2 (25 U/ml). After 24 h of incubation, supernatant was recovered and IFN-γ levels were determined. As reported in figure 7.7, IFN-γ production was detectable only in the presence of N202.1A tumour cells (a).

Since the N202.1A cell line is characterised by the overexpression of the *neu* oncogene, it was of interest to investigate whether the production of IFN-γ was restricted to the specific recognition of the oncogene. Indeed, it was presumed that immunisation with tumour cells overexpressing HER2/neu oncoprotein transduced with *Ag38* gene might have induced the break of the tolerance to the transgene.

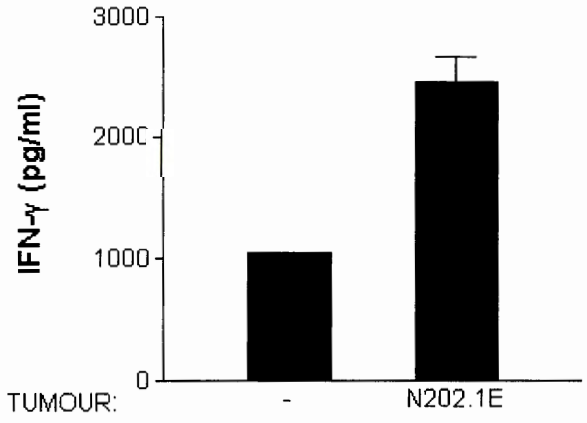
Therefore, the production of IFN-γ was evaluated after stimulation with the N202.1E line, an independent FVB-NeuN derived spontaneous tumour cell line characterised by the lack of expression of HER2/Neu oncoprotein.

As reported in figure 7.7, IFN-γ production was detectable also in the presence of N202.1E tumour cells (b).

In summary, vaccination with *Ag38*-transduced N202.1A tumour cells induced the activation of a prevalent type 1 $CD8^+$ T cell population producing IFN-γ. The production of this cytokine was observed in response to stimulation with non-transduced tumour cells and this response was not specifically restricted to stimulation with HER2/neu-positive tumour.



a



b

Fig. 7.7: Production of IFN-γ by activated lymphocytes in response to stimulation with tumour cells

IFN-γ production by splenocytes obtained from mice vaccinated with *Ag38*-transduced cells, measured in supernatant of lymphocytes cultured alone or in the presence of N202.1A (**a**) or N202.1E (**b**) tumour cells.

7.8 INHIBITION OF TUMOUR PROLIFERATION BY IFN-γ

A direct role for some cytokines in inhibition of tumour proliferation has been reported in a number of normal and malignant cells (Burke et al., 1999; Gooch et al., 1998).

To assess whether IFN-γ produced by vaccination treatments might have a direct growth inibitory effect on spontaneous mammary tumour in FVB-NeuN mice, the sensitivity of different transgenic tumour cell lines to IFN-γ was evaluated *in vitro*.

Cultures of the three different tumour cell lines N202.1A, N202.1E and TT3, each derived from a mammary carcinoma spontaneously grown in FVB-neuN transgenic mice, were incubated with a range of concentrations of the cytokine. *In vitro* proliferation of cells was determined using an SRB (sulforhodamine B) assay.

As shown in figure 7.8, after 5 days of incubation with IFN-γ, even at the very low dose, a strong decrease of the proliferation index of cells was observed in all the different cell lines.

The strong inhibitory effect of this cytokine on the three different transgenic tumour cells suggest that induction of this cytostatic cytokine at the tumour site might represent at least one mechanism of action of the vaccine.

7.9 SUMMARY

The efficacy of vaccine obtained by transduction of a mammary tumour cell line with the *M. tuberculosis Ag38* gene, was investigated in female mice transgenically expressing the rat *HER-2/neu* protooncogene. These mice spontaneously develop stochastic mammary tumours after a long latency period.

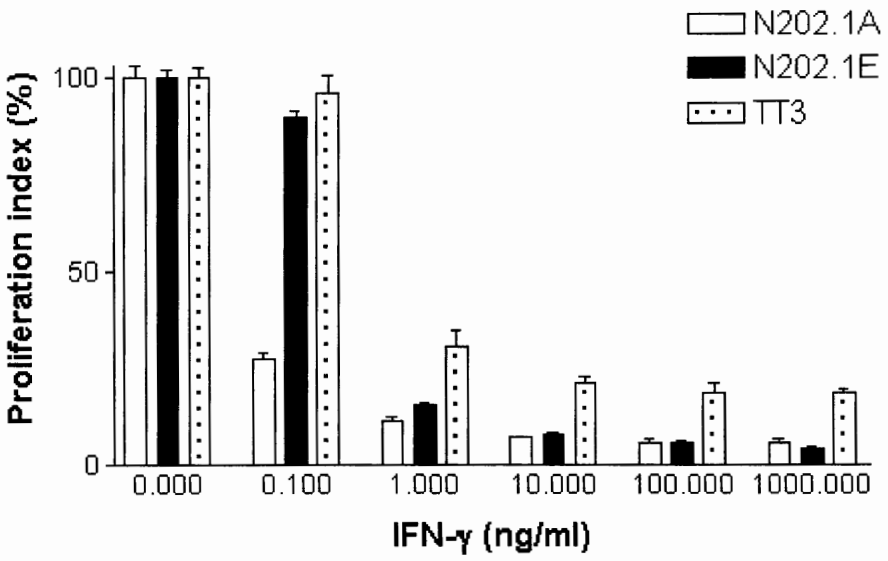


Fig. 7.8: Direct effect of IFN-γ on proliferation of transgenic mice-derived tumour cell lines

Effect of IFN-γ on proliferation of N202.1A, N202.1E and TT3 tumour cells. Proliferation index is expressed as a percentage of absorbance in the absence of IFN-γ. Data are mean ± S.D. of quadruplicate determinations.

The onset of spontaneous mammary tumours was significantly delayed in mice vaccinated with *Ag38*-transduced cells, but not in mice vaccinated with non-transduced cells as compared with untreated mice. Protection from spontaneous tumour development was increased when mice were vaccinated with the mycobacterium-gene-transduced vaccine plus a systemic administration of IL-12 at a low dose. Mice vaccinated with non-transduced cells plus IL-12 developed tumours, with only a slight delay in tumour appearance as compared with the control group.

Lymphocytes obtained from lymph nodes of mice vaccinated with transduced cells secreted high levels of IFN-γ. This secretion was not mainly dependent from a IL-12-mediated stimulation.

An activated $CD3^+$ $CD8^+$ T cell population was evoked in mice vaccinated with transduced cells. This population responded to tumour cells with IFN-γ production, which was not specifically restricted to HER2/Neu oncoprotein expression.

These data indicate the efficacy of a short-term protocol of vaccinations exploiting the adjuvant potency of a *M. tuberculosis* gene and low doses of IL-12 in a model of stochastic development of mammary tumours.

CHAPTER EIGHT

CHARACTERIZATION OF IMMUNE RESPONSE IN MICE PROTECTED FROM SPONTANEOUS MAMMARY CARCINOMA

Further analyses were performed to investigate the immunological effectors activated in protected mice. These studies were performed in two mice completely protected from spontaneous tumour development, gained as a result of the administration of *Ag38*-cell vaccine plus IL-12 as the most effective protocol.

8.1 SEROLOGICAL RESPONSE

Based on the results obtained in the melanoma model, it was of interest at first to evaluate whether an humoral response against tumour was induced in mice protected from spontaneous tumour development.

All serum samples from mice in each experiment were collected after the second vaccination treatment with *Ag38*-transduced or non transduced cells, at different time during the tumour latency period and, in the case of mice completely protected, at different times till the end of the observation period. Sera from untreated mice were collected as control.

Flow cytometry analysis was used to evaluate the presence in these sera of antibodies directed against antigens expressed on N202.1A mammary carcinoma cells. The analyses performed on some representative serum samples are shown in Fig 8.1.

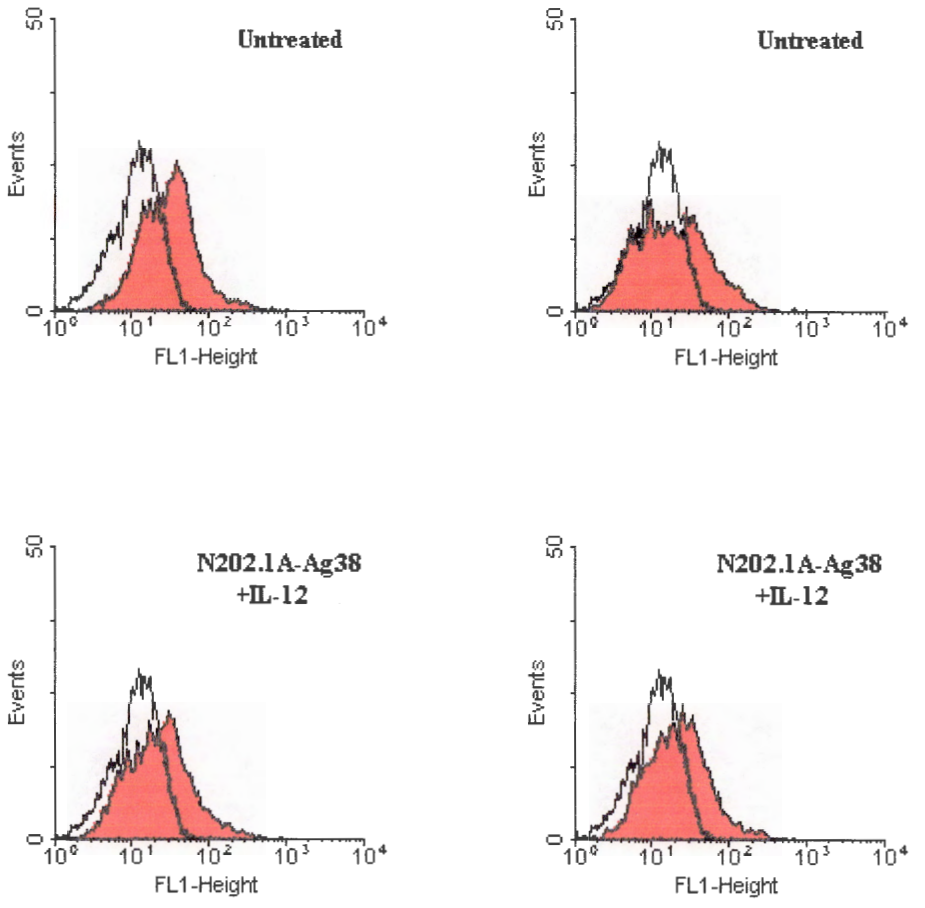


Fig. 8.1: Humoral response against parental N202.1A cells in mice protected from spontaneous tumour

Reactivity against N202.1A-associated antigens in some representative serum samples from mice vaccinated with *Ag38*-transduced cells plus IL-12 protected from tumour growth (N202.1A-Ag38+IL-12) or from not vaccinated mice (untreated). FACScan analysis was performed on N202.1A cells, incubated with serum diluted 1:10 and stained by an indirect method using goat FITC-anti-mouse IgG+IgM. Open areas indicate cells stained with secondary antibody alone

No significant increase in antibody titre directed against N202.1A-associated antigens was observed in sera of mice completely protected from tumour development neither immediately after vaccination nor during the period of the experiment.

Therefore, unlike the situation observed in the melanoma model, in which mice protected from parental tumour challenge developed a strong humoral response against tumour, in the spontaneous tumour model no production of antibodies directed against tumour cells was detectable.

8.2 CELLULAR RESPONSE

The activation of a cellular response was then evaluated. To this aim, spleen cells of the two mice completely protected from tumour development were removed and cultured *in vitro* with stimulation of irradiated N202.1A tumour cells, IL-2 at low dose (25 U/ml) and syngeneic irradiated splenocytes (feeder cells).

To select an activated population able to respond to the tumour, these cells were maintained *in vitro* for four weeks with weekly restimulations as above.

Further analyses were therefore performed to characterise the cell population able to survive in this prolonged culture *in vitro*.

8.2.1 Characterisation of *in vitro* long-survived cell population

The proliferation activity of these cells in the presence of N202.1A tumour cells was determined. To this aim, cells were co-incubated for 72 hours with irradiated N202.1A tumour cells, IL-2 at low dose and with or without syngeneic irradiated splenocytes as feeder. Cultures were then pulsed with ^{3}[H]thymidine and proliferation was estimated after 8 hours. Splenocytes obtained from naïve mice were incubated in the same conditions and used as control. The result of the experiment is reported in table 8.1.

	Cells from a protected mouse (A)	Cells from a protected mouse (B)	Control splenocytes
Alone	16967 ± 2044	15797 ± 8499	9832 ± 938
+ Tumour	17841 ± 727	10754 ± 1184	12920 ± 4906
+ Feeder and Tumour	83714 ± 13902	52309 ± 1705	15955 ± 9014

Table 8.1: Proliferation of *in vitro* long-survived splenocytes from protected mice

Proliferation of cells obtained from two protected mice, after *in vitro* re-stimulations with N202.1A tumour, IL-2 and feeder cells. Data, calculated as mean ± S.D. of triplicate determinations, represent [^{3}H] Thymidine uptake (cpm) of cells cultured alone, or in presence of N202.1A tumour cells, or in presence of N202.1A tumour cells and feeder cells. Proliferation activity of splenocytes obtained from a naïve mouse was evaluated as control.

As compared to control splenocytes, cells obtained from mice completely protected from spontaneous tumour showed a higher proliferation activity in the presence of feeder cells.

These data, therefore, suggest that the activation of this tumour-responding population might be increased by the presence of a subset of immune cells, as a component of feeder cells.

In vitro assays were then performed to investigate the cytotoxic activity of these cell populations against the tumour. After incubation with N202.1A tumour cells labelled with ^{51}Cr, a standard 4-h ^{51}Cr release assay was performed.

No cytotoxic activity against tumour cells was detected (less than 6% of lysis percentage) in these conditions. Therefore, the cell population derived from mice completely protected from spontaneous tumour were not able to lyse N202.1A tumour cells *in vitro*.

The type of immune cell subset derived from protected mice was then investigated by FACScan analyses.

No Natural Killer (NK) cells were present in this immune cell-population, as revealed by a first analysis performed using an antibody directed against the NK1.1 surface antigen (Fig 8.2A)

An analysis to evaluate the presence of activated $CD4^+$ or $CD8^+$ lymphocytes and their cytokine production was then performed. To this aim, five days after the weekly re-stimulation, cells were cultured for 21 h with immobilised anti-CD3 mAb and 1 μM monensin, added for the last 12 h to inhibit protein transport. Surface marker and intracellular cytokine staining was performed using Cy-Chrome-labelled anti-CD8 or anti-CD4, FITC-anti-IFN-γ and PE-anti-IL-4 mAbs. As shown in figure 8.2B, this analysis revealed the presence of mainly a $CD3^+$ $CD4^+$ population producing IL-4.

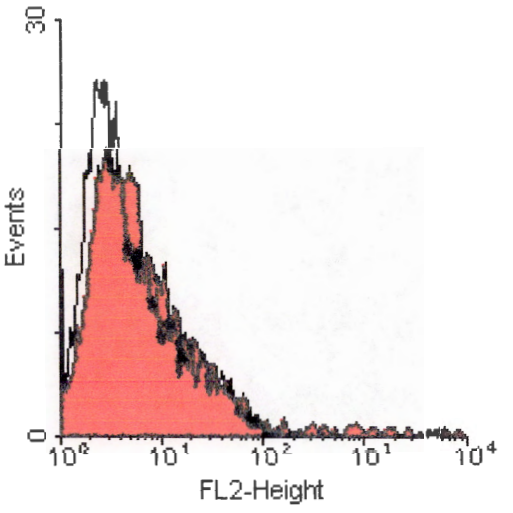


Fig. 8.2A: Characterisation of *in vitro* long-survived splenocytes by FACScan analysis for the presence of NK cells

Characterisation of splenocytes derived from mice protected from spontaneous mammary tumour and survived to prolonged *in vitro* culture in presence of N202.1A tumour cells. Flow cytometry analysis of cells stained with PE-anti-NK1. Open areas indicate cells stained with a PE- antibody isotype control.

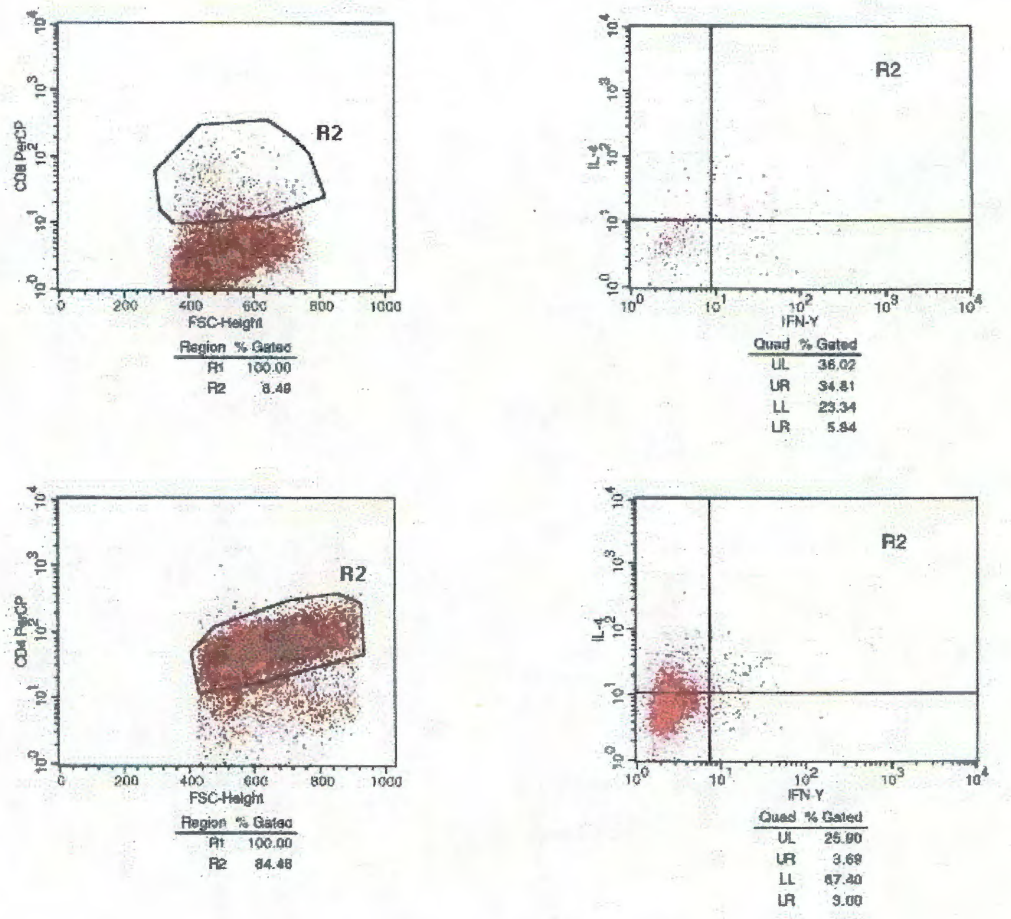


Fig. 8.2B: Characterisation of *in vitro* long-survived splenocytes by FACScan analysis for the presence of $CD4^+$ and $CD8^+$ cells

Characterisation of splenocytes derived from mice protected from spontaneous mammary tumour and exposed to prolonged *in vitro* culture in presence of N202.1A tumour cells. Flow cytometry analysis of cells stimulated with immobilised anti-CD3 mAb, in the presence of monensin, and triple-stained with Cy-Chrome-anti-CD8 or Cy-Chrome anti-CD4, PE-anti-IL-4 and FITC-anti-IFN-γ mAbs. Analysis of cytokines production was performed setting a gate on CD8- (top) or CD4- (bottom) positive cells.

8.2.2 Inhibition of *in vitro* tumour growth

The ability of this activated cell population to directly inhibit tumour growth through the release of inhibitory factor(s) was then evaluated.

To this aim, the sensitivity of different transgenic tumour cell lines to supernatant obtained from culture of these CD3+CD4+ cells was analysed *in vitro*. Cultures of the three different tumour cell lines N202.1A, N202.1E and TT3, each derived from a mammary carcinoma spontaneously grown in FVB-neuN transgenic mice, were incubated with supernatant diluted 1:2 recovered three days after the weekly re-stimulation of $CD3^+CD4^+$ culture. After four days of incubation, *in vitro* proliferation of cells was determined using an SRB (sulforhodamine B) assay.

As shown in figure 8.3, a decrease, which was very strong in N202.1A and TT3 cells, of the proliferation index was observed in all three transgenic cell lines, while no inhibition was detected in the growth of an unrelated murine control cell line.

As with other cytokines, IL-4 has been reported to inhibit proliferation of some tumour cell lines (Burke et al., 1999; Gooch et al., 1998). Therefore, since, as revealed by FACScan analysis, this cell population produced IL-4, it was investigated whether the inhibitory effect observed on the growth of transgenic tumour cells was mediated by this cytokine.

As shown in figure 8.4, a SRB assay performed after incubation of N202.1A cells in the presence of IL-4 did not reveal any inhibitory effect, even at high dose.

Therefore, the $CD3^+CD4^+$ cell population was demonstrated to directly inhibit *in vitro* tumour growth through the release of inhibitory factor(s). This inhibition was not mediated by the release of IL-4.

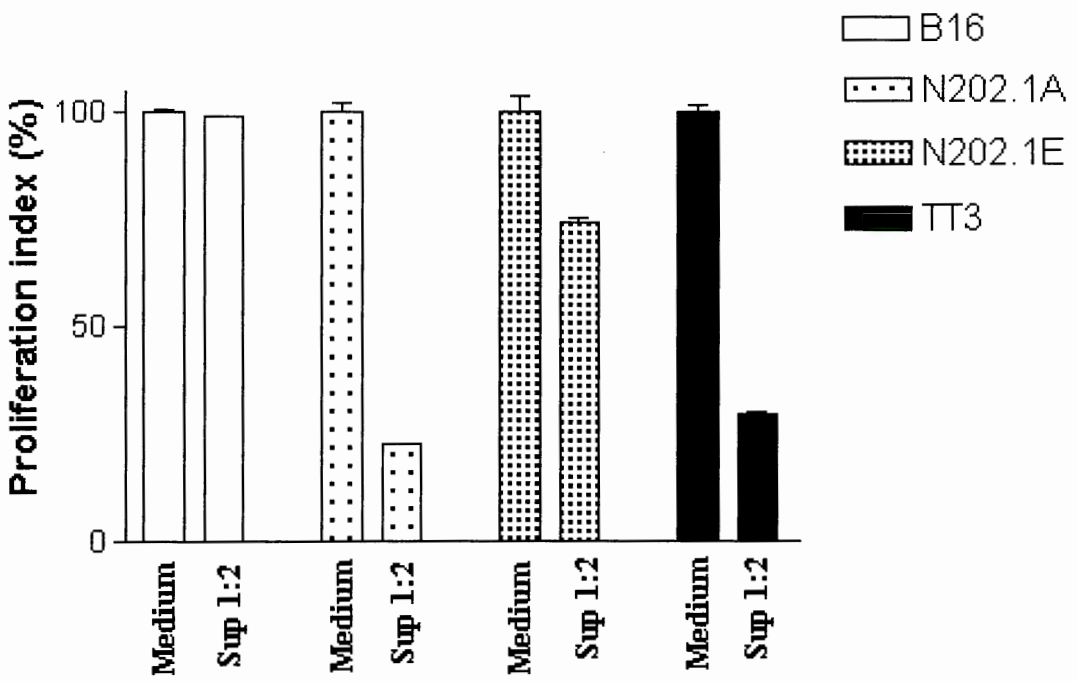


Fig. 8.3: Inhibition of *in vitro* tumour growth by supernatant of long-survived splenocytes

A representative experiment showing the sensitivity of N202.1A, N202.1E, TT3 and B16 tumour cells to supernatant obtained from culture of $CD3^+CD4^+$ cell population, diluted 1:2 (Sup 1:2). Proliferation index is expressed as a percentage of absorbance in the presence of medium alone (Medium). Data are mean ± S.D. of quadruplicate determinations.

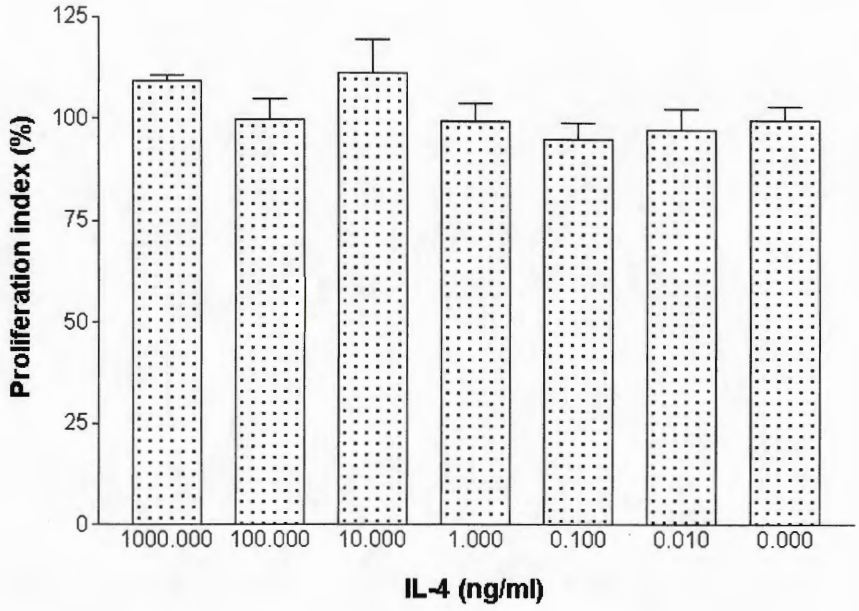


Fig. 8.4: Direct effect of IL-4 on proliferation of N202.1A cell line

Effect of IL-4 on proliferation of N202.1A tumour cells. Proliferation index is expressed as a percentage of absorbance in the absence of IL-4. Data are mean ± S.D. of quadruplicate determinations.

8.2.3 Passive transfer of immunity (Winn test)

In view of the inhibition of *in vitro* tumour growth observed in the study above, an experiment was then performed to evaluate whether the activated $CD4^+$ T cell population transferred *in vivo* in mice could be able to protect from a tumour challenge. After expansion of the culture, CD4+ T cells were subcutaneously injected to naïve animals mixed with live N202.1A tumour cells. A control group received an injection of N202.1A cells in the presence of splenocytes obtained from a naïve mouse *in vivo* depleted of the CD8 T cell population (Fig. 8.5) and *in vitro* depleted of adherent cells by passage over a nylon wool column. Four FVBNeuN mice were treated in each group and a ratio of 30:1 between immune cells (3×10^6) and live tumour cells (1×10^5) was administered in each animal. Mice were then monitored for the development of a subcutaneous tumour at the site of injection.

As shown in Figure 8.6, no differences were observed in the development of tumour mass between mice injected with activated CD4+ T cells and control mice injected with CD8-depleted splenocytes. No differences in the number of tumour-infiltrating lymphocytes were also detected by histologic evaluation between the two groups of mice (data not shown).

8.3 SUMMARY

The immunological effectors activated in mice protected from spontaneous mammary tumour development were investigated. No production of antibodies directed against tumour cells was detectable, while a cell population able to survive to *in vitro* culture was derived from these mice. These immune cells, able to proliferate in the presence of tumour cells and lacking an *in vitro* cytotoxic activity, were characterised as CD4+ T

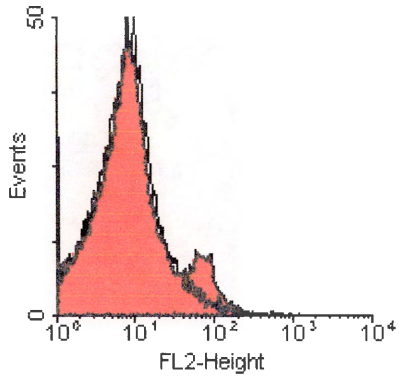


Fig. 8.5: FACScan analysis of *in vivo* CD8-depleted splenocytes

FACScan analysis with murine FITC-anti-CD8 antibody on splenocytes from FVBNeuN mice depleted of $CD8^+$ T lymphocytes (overlay) or non-depleted control mice (filled), performed to verify the depletion of the appropriate subset

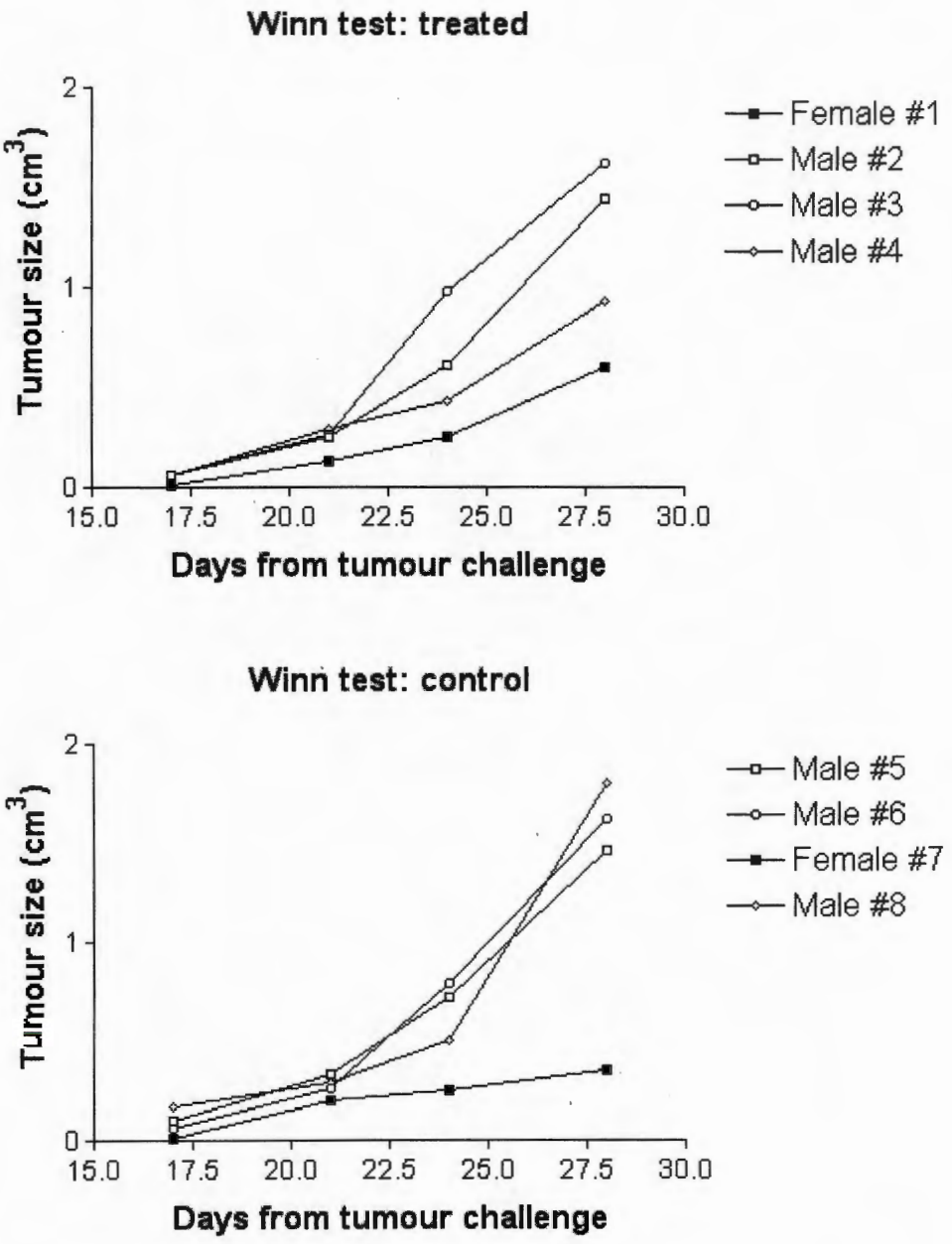


Fig. 8.6: Passive transfer of immunity (Winn test)

Growth of subcutaneous injected N202.1A tumour in mice transferred with the $CD4^+$ T cell population (treated) and in mice transferred with control cells (control). Four mice, 3 males and 1 female, were treated in each group.

cells producing IL-4. A direct inhibition on the growth of FVBNeuN transgenic tumour cell lines mediated by the release of inhibitory factor(s) was observed *in vitro*. This effect was not mediated by the release of IL-4, since this cytokine did not inhibit the *in vitro* growth of the transgenic tumour cell lines.

However, this cell population transferred *in vivo* in mice failed to protect against a subcutaneous tumour challenge.

CHAPTER NINE

DISCUSSION

The identification in murine and human tumours of various antigens representing different histiotypes that serve as potential targets for an immune response, has provided an opportunity to develop new, and hopefully more effective, anticancer therapies. These antigens, derived from endogenously synthesised proteins, are seen as foreign by the host, either as a consequence of mutations that arise during malignant transformation or because they represent gene products not normally expressed in adult tissues.

The immune system is capable of recognising and responding to tumours via several effector mechanisms. Among these different mechanisms, the CTL response seems to be the most important; indeed, some data demonstrate that CTLs can be generated which specifically recognise tumour antigens presented on the cell surface together with MHC class I molecules. However, generally the CD8+ T cell response is likely to be inadequate, and strategies for active immunotherapy must attempt to recruit alternate cytolytic effector cells (Pardoll, 1996).

Mitchinson (1970) hypothesised that the combination of poorly antigenic and highly antigenic determinants on a common carrier would augment the immune response toward the poorly antigenic determinants by stimulating immune recognition (Kim et al., 1992). On the basis of this theory, the thesis explores the use of a gene encoding an highly antigenic protein, transduced in tumour cells, in increasing the immune response against weak tumour antigens.

9.1 TUMOUR MODELS

I chose to study two murine tumour models which, like human tumours, although they express tumour antigens demonstrated poor immunogenicity. Immunogenicity refers to the ability of a tumour to elicit an immune response against itself, that can be evaluated by resistance against a challenge with live tumour cells following vaccination with irradiated tumour cells.

The first tumour chosen, B16 murine melanoma, expresses different tissue-specific differentiation antigens (Figure 5.8). These antigens correspond to those expressed on human melanoma, which have been reported to represent potential targets for an immune response in patients. Moreover, like many advanced tumours and metastases in humans, murine B16 melanoma express very low level of MHC class I antigens.

As a second tumour model, I have chosen a mammary carcinoma tumour that spontaneously grows in *HER2/neu* transgenic mice. This tumour, although expressing the potential antigenic target HER2/neu oncoprotein, demonstrated a poor immunogenicity. About 30% of human breast carcinomas overexpress HER2 oncoprotein. An antibody response specifically directed against HER2 oncoprotein has been detected in some human breast carcinoma patients (Pupa et al., 1993; Disis et al., 1997); however the majority of patients seem to be tolerant to the antigen.

Under the experimental conditions used in this thesis to evaluate protection against melanoma or spontaneous mammary carcinoma by vaccination with tumour cells, parental B16 and N202.1A cells show a very poor immunogenicity. Indeed, the majority of mice vaccinated with parental irradiated cells developed tumours (Figure 4.2 and 7.2) with not significant difference when compared to control not vaccinated mice.

9.2 GENE TRANSFER AND EXPRESSION OF AG38 MOLECULE IN TUMOUR CELLS

In an effort to elicit an immune response against tumour antigens, I decided to transduce tumour cells with a gene of *Mycobacterium tuberculosis*. In less than a decade the archetypal view that the immune system exists primarily to distinguish "self" from "non-self" has been replaced by the paradigm that the immune system functions primarily to distinguish "dangerous" from "non-dangerous" antigens (Matzinger, 1994). Presumably the immune system has evolved over millions of years to respond to bacteria with a rapid activation of defences that are best suited to fight microbial infection. Because *Mycobacterium tuberculosis* is a major target in this fight, mycobacterial molecules are promising candidates to be used as adjuvants for the development of effective therapeutic or prophylactic tumour vaccines. The immune response elicited by mycobacteria might facilitate activation of the immune system against tumour antigens and result in the eventual selective destruction of tumour cells through a specific immune response. Indeed studies on complete Freund's adjuvant (CFA) indicate that mycobacteria contain several substances that stimulate the immune response and promote Th1 differentiation (Forsthuber et al., 1996). Moreover, studies in different countries have shown that neonatal Bacillus Calmette-Guérin (BCG) vaccination confers some degree of protection against leukaemia and other childhood cancers (Grange et al., 1995) and recently a significant reduction in melanoma risk in people vaccinated with vaccinia virus or BCG was reported (Koelmel et al, 2000). However, the use of BCG as immunoadjuvant in patients with different cancer types has produced variable results (Grange et al., 1995). Such variable results are also observed when BCG is used in vaccination against tuberculosis (Fine PEM, 1994). There is some evidence that a similar mechanism underlies the efficacy of BCG in therapy and in

prophylaxis against tuberculosis and tumour prevention (Grange et al., 1990). The BCG efficacy appears to depend on whether a predominant Th1- or a Th2-type cytokine secretion pattern is induced. Several findings, indeed, have suggested that protective immunity to infection is associated with pronounced Th1 lymphocyte expansion and activity with increased production of cytokines typical of this subset. Conversely the enhancement of Th2 function is associated with decreased Th1 activity and progression of disease or infection (Heinzel et al., 1989; Pearce et al., 1991).

One hypothesis for these conflicting results is that BCG could contain both substances able to induce a protective immune response and substances which stimulate the secretion of immunosuppressive cytokines depending on dose, schedule and the individual subject (Fine et al., 1990). Therefore I postulated that using a product of a single gene, instead of the whole bacillus, it might be possible to enhance Th1 cell maturation pathway, avoiding the suppressive effects observed in some patients using the whole bacillus.

To create the cell vaccine I chose the *Mycobacterium tuberculosis Ag38* gene. Indeed, the 38 kDa antigen is known to be an immunodominant antigen during the development of the immune effector mechanism against tuberculosis (Kadival et al., 1987; Verbon et al., 1992). This antigen has been demonstrated to evoke the generation of a Th1-type response preferentially, as revealed by the secretion of Th1-like lymphokines (IL-2 and IFN-γ) and by the preferential production of 38 kDa specific IgG2a-type antibody (Agrewala et al., 1995).

Furthermore, analysis of *Ag38* gene sequence revealed the presence of 14 CpG motifs, 8 of which are maintained in the open reading frame region used to transduce tumour cells (Fig. 3.1). CpG motifs, consisting of CpG dinucleotides within specific flanking bases, are under-represented and selectively methylated in vertebrate DNA, but are present at higher frequency and unmethylated in bacterial DNA. DNA containing these

motifs has been reported to induce a rapid direct activation of macrophages to secrete IL-12 (Krieg et al., 1998), resulting in the final activation of a Th1-type immune response.

To transduce this gene in tumour cells, I initially chose a PRC/CMV eukaryotic vector and conventional transfection strategies. As compared to the use of vectors based on retroviruses, I considered these strategies likely to be safer in the context of their use in humans. Indeed, although retroviral vectors are extraordinarily efficient gene delivery vehicles, the inadvertent production of replication-competent retroviruses and the possibility of insertional mutagenesis due to their integration in the eukaryotic genome, even if these are very unlikely events, may limit their application in humans. However, using the conventional strategies, I failed to achieve good results. Not only were tumour cells expressing the bacterial protein obtained with a low efficiency but also the expression of the bacterial product in transfected tumour cells was low and very unstable (data not shown).

As a consequence of these preliminary studies a retroviral vector, modified to express the leader and transmembrane portion of Nerve Growth Factor Receptor (NGFR) and the stop codon region of human IL-3, was then constructed for my purpose. This modification resulted in the transduction of tumour cells with high efficiency and resulted in the stable expression of Ag38 bacterial protein in both tumour models. Furthermore, due to the presence of the NGFR transmembrane sequence, Ag38 protein was expressed on the tumour cell surface, allowing me to detect and tag transduced cells.

9.3 *AG38* TRANSDUCTION IN THE VACCINATION AGAINST MELANOMA

At a dose of 2.5×10^5 cells, which induced the subcutaneous growth of tumour in 100% of mice injected with parental cells, only 60 % of mice injected with *Ag38*-transduced cells developed tumours (Fig. 4.1). Moreover, compared to parental cells, I observed a delay in the appearance of tumour and no metastases developed during the observation period.

Significant protection against tumour growth after subcutaneous or intravenous injection of parental melanoma tumour cells has been observed in mice vaccinated with *Ag38*-transduced melanoma cells. Indeed, as an overall result from all the experiments performed, it was observed that 80 % of mice injected subcutaneously were completely protected from tumour growth and 20-30 % of mice intravenously injected failed to develop lung metastases. Moreover, a response to *Ag38*-cell vaccine was also observed in mice which developed tumours, since in these mice the onset of tumour growth, as well as the mortality, was delayed in comparison with non-immune animals and animals immunise with non-transduced-cells (Fig. 4.2 and 4.3).

Regarding the immune mechanism induced by the vaccine, the presence of Ag38 protein does not seem to work as a chaperone molecule by influencing the ability of melanoma cells to present endogenous antigens, as has been observed using tumour cells transduced with mycobacterial genes encoding heat-shock protein (HSP). Indeed, vaccination with tumour cells transduced with HSP 65 from *Mycobacterium leprae* (Lukacs, 1993) and *Mycobacterium bovis* (Schweighoffer, 1996) has been reported to achieve enhancement of the immune response against tumour antigens, by enhancing the expression of MHC class I surface antigens. The HSPs are molecular chaperones that mediate the assembly and folding of other proteins (Hartl, 1996).

They are also associated *in vivo* with the entire repertoire of peptides generated within the cell, and these non-covalent HSP-peptide complexes are particularly immunogenic (Blachere et al., 1995). The B16 cell lines used in this thesis express very low or undetectable levels of MHC class I antigens *in vitro*. No increase in the expression of MHC class I molecules has detected after transduction of the *Ag38* gene into melanoma cells (data not shown). Therefore, the effect of *Ag38* in this context differs from that observed using HSP 65.

Vaccination with *Ag38*-transduced melanoma cells induced a preferential Th1 response (Tab 4.1) The induction of a Th1 cytokine secretion pattern by endogenously expressed mycobacterial antigen is consistent with results reported by Huygen et al. (Huygen et al., 1996) and Zhu et al (Zhu et al., 1997) who, in their studies on tuberculosis vaccines, showed that vaccinations with plasmid DNA constructs encoding the 85T or Ag38 *Mycobacterium tuberculosis* antigens are a powerful approach to generate a specific helper response with a Th1-like phenotype. A Th1-type response might be important in the control of tumour growth. Indeed, a Th1 response may be effective in activating NK cells, cytotoxic T lymphocytes and tumouricidal macrophages, thus increasing the killing of tumour cells (Sredni et al., 1996).

The induction of a Th1-type response might depend on more than one factor. For example both the expression on the tumour cell surface of the strong mycobacterial antigen and the presence of CpG immunostimulatory motifs in the Ag38 encoding gene. The presence of mycobacterial protein on the cell surface of irradiated tumour cells can improve their uptake by activated antigen-presenting cells (APC), which might introduce endocytosed-tumour antigens into both the MHC class II and class I processing pathways, effecting cross-priming with weak tumour antigens (Gromme et al., 1999; Heath et al., 1999). The CpG motifs flanked 5' by two purines and 3' by two pyrimidines have a potent immunostimulatory activity. Prokaryotic and vertebrate DNA

differ in the relative abundance of CpG-dinucleotides and the degree of cytosine methylation raising the possibility that these structural differences are used by immune cells to discriminate pathogen-derived "dangerous", from "self", DNA. Bacterial DNA containing CpG-sequences has been observed to activate antigen-presenting cells (APCs) such as macrophages and dendritic cells (Zimmermann et al., 1998; Pisetsky, 1997; Sparwasser et al., 1997; Sparwasser et al., 1997; Sparwasser et al., 1998) upon DNA endosomal uptake, resulting within minutes in activation of the stress kinase pathways (Hacker et al., 1998) and NF-kB (Sparwasser et al., 1997; Stacey et al., 1996). As a consequence APCs produce cytokines, including IL-12, IL-6, IL-1 and TNF-a, and upregulate co-receptor molecules (Sparwasser et al., 1998). Recent work has demonstrated the powerful adjuvant effect of CpG-ODNs, which can be used to trigger protective and curative Th1 responses *in vivo* (Zimmermann et al., 1998; Lipford et al., 1997; Lipford et al., 1997; Chu et al., 1997). Therefore, the strong immune response against mycobacterial epitopes and CpG motifs might create a microenvironment highly concentrated for cytokines, which promotes the activation of minor clonotypes directed against weak tumour antigens. In this hypothesis the *Ag38* gene might play the role of adjuvant, increasing the immune activation and promoting the recognition of poorly immunogenic tumour cell antigens, finally resulting in a specific anti-tumour immune response. The property of allowing the recognition of the poorly immunogenic murine melanoma B16 seems to be a characteristic of bacterial products, since vaccination with ovalbumin-transfected melanoma does not induce a protective response to non-transfected parental melanoma B16 (Falo, Jr. et al., 1995).

The expansion of clonotypes directed against tumour antigens induced by vaccination might promote a rapid immune response against the live non-transduced tumour administered after vaccinations. A concerted response involving the activation of different populations of immunocytes has to be postulated. Indeed, the depletion of CD8

T lymphocytes evaluated in the *in vivo* experiment (Figure 4.4) did not reduce the protection induced by vaccination with transduced cells.

Mice vaccinated twice with irradiated transduced B16 cells exhibit low serum antibody titres. However, mice immunised with transduced B16 cells and protected from challenge with parental B16 melanoma cells showed a strong humoral response against the parental cell antigens which was detectable at serum dilutions up to 1:1000 (Fig. 5.3). Most of these antibodies were of an IgG2a isotype, which represents the main IFN-γ- dependent isotype in mice (Fig. 5.4) (Snapper et al., 1993). Therefore, the prevalent isotype observed during the humoral response after challenge with viable cells is consistent with the Th1-type response induced by the vaccine.

A positive contribution of antibodies to tumour protection was indicated by *in vitro* results showing that antibodies present in the sera of mice surviving the challenge mediated cytotoxic effects on parental melanoma cells through complement activation. Moreover, *in vivo* passive immunisation with a pool of sera from protected mice resulted in a decrease in the number of experimental B16 lung metastases (Fig. 5.5 and 5.6). These findings, although obtained in a small number of mice, suggested the possible involvement of antibodies in tumour protection, but do not exclude a role for cytotoxic T cells. As the cellular and humoral immune systems work in concert, it would be surprising if cancer-associated antigens would induce only a cellular or humoral response. Indeed, in humans the frequency of CTL precursors specific for melanoma antigens has been observed to be usually very low ($<1/10^5$). Therefore, in patients immunised with tumour antigens a CTL response has been detected only after *in vitro* expansion of specific T cells by 1 to 3 restimulations with specific antigen (Kawakami et al., 1998). The serological response of the mice was directed predominantly to products encoded by endogenous murine leukaemia viruses (Fig. 5.11,

5.12). These findings, in light of the high levels of viruses expressed on viable B16 cells (Li et al., 1998), are consistent with previous data (Leong et al., 1988).

A serological response against TRP-2, the antigen found by screening a cDNA library from B16 to be the major target recognised by tumour-reactive T cell clones (Bloom et al., 1997), has not been detected (Fig. 5.14, 5.15 and 5.16). Moreover, with in the limitations of Western blot analysis, no antibodies directed against other melanocyte lineage-specific antigens, such as tyrosinase, TRP-1 and gp100, were found (Fig. 5.17). However, it cannot be excluded that the presence of low amount of antibodies against these antigens might be detectable by serological analysis of recombinant cDNA expression libraries (SEREX) of B16 melanoma cells using immune serum (Sahin et al., 1995). Since the presence of antibodies against melanocyte lineage-specific antigens has been observed in some melanoma patients (Huang et al., 1998; Okamoto et al., 1998), it is plausible that, in the B16 murine model, their immune recognition is inhibited or masked by the presence of viral antigens.

In order to enhance the anti-tumour immune response, the effect of re-administration of irradiated transduced tumour cells (recall) in vaccinated mice immediately after tumour challenge was evaluated. Although this study constituted only a single experiment, it provided an unexpected result. Thus a detrimental effect was observed during this experiment, since a reduction of tumour protection induced by vaccination was observed (Fig. 6.4). The mechanism to explain the adverse effect of re-administration of the vaccine after tumour challenge was not investigated.

Irradiated *Ag38*-transduced cells administered three times, on days +1, +8 and +15 from tumour challenge, did not have a therapeutic effect on existing tumour (Fig. 6.5). The ability of cancer vaccines to cure existing tumours has been intensively investigated, but with limited successes. In most studies, only a minority of tumour-bearing mice were

cured, and this limited efficacy was achieved solely when the vaccine was administered in the first few days after challenge (Cavallo et al., 1997). This limited efficacy in curing existing tumours is thought to rest primarily in inadequate penetration of tumour mass by the immune cells and in the escape of some tumour cell progeny from the immune response. Moreover, presumably, the rapid replication of the live injected tumour cells in the experimental models leads to the death of mice before the activation of an efficient immune response.

Different vaccination strategies based on the use of fractions of materials derived from *Mycobacterium tuberculosis*, administered as a general immunostimulant or as an adjuvant together with tumour cells, have been evaluated and compared to the use of the Ag38-expressing cellular vaccine.

Recombinant Ag38 protein was administered exogenously in incomplete Freund's adjuvant or, in order to maintain the protein in the same context of melanoma antigens, in the presence of irradiated non-transduced melanoma cells. A low protection against tumour challenge was induced by these vaccination treatments (Fig. 6.1). The administration of DNA plasmid encoding the Ag38 antigen was evaluated as an alternative strategy. A very low protection against tumour challenge was induced by this vaccination (Fig. 6.2). Administration of whole live Bacillus Calmette-Guérin as an immunostimulant was also evaluated. A delay in tumour growth was induced in mice immunised with BCG before the tumour challenge, as compared to control mice which received only tumour challenge (Fig. 6.3). However, the level of protection observed in these experiments was less than that evoked by vaccination with *Ag38*-transduced cells. It is plausible that, using a tumour cell endogenously expressing the mycobacterial protein, a response against the tumour cell antigens expressed in the same context might also be increased, thus resulting in a more selective destruction of tumour cells.

9.4 *AG38* TRANSDUCTION IN VACCINATION AGAINST A MAMMARY CARCINOMA SPONTANEOUSLY GROWING IN TRANSGENIC MICE

The recent discovery of gene mutations that predispose to cancer now enables identification of at-risk individuals with a defined genetic prognosis (Jonsen et al., 1996). The goal of vaccination in such individuals is the recruitment of the immune system to eliminate single transformed cells before tumour nodules develop. In this thesis the efficacy of the *Ag38*-cancer vaccine was evaluated in proto-*neu* transgenic mice. In this strain, the expression of proto-neu induces the development of spontaneous focal mammary tumours in all females (Guy et al., 1992) although the stochastic development of the tumours and the long latency period indicate the requirement for additional events in tumour formation.

Vaccination of proto-*neu* transgenic mice with *M. tuberculosis Ag38*-transduced tumour cells resulted in a significant delay in tumour onset (Fig. 7.1 and 7.2). Consistent with previous data obtained in the experimental melanoma tumour model, endogenously expressed mycobacterial antigen in tumour cell vaccine induced a preferential Th1 response (Fig. 7.4).

As reported above, the DNA of the *M. tuberculosis Ag38* gene used for transduction contains 8 CpG motifs. Such CpG DNA motifs have been shown to activate monocytes and macrophages directly to secrete cytokines, especially IL-12 (Klinman et al., 1996; Krieg et al., 1998; Chu et al., 1997). Consistent with these findings, I found detectable levels of serum IL-12 in two of five mice after vaccination with *M. tuberculosis*-transduced (data not shown). It was investigated whether a systemic co-administration of rIL-12 together with the non-transduced tumour cells could induce the same anti-tumour response observed using the transduced tumour cell vaccine. Indeed, an adjuvant effect of IL-12, with induction of protective cell-mediated immunity, in a

vaccine against Leishmania has been described (Afonso et al., 1994), as well as an increase in both humoral and cell-mediated immune responses in a vaccine against schistosomes (Wynn et al., 1996). In oncology, used alone, IL-12 has been shown to be effective against many murine tumours (Brunda et al., 1993; Rodolfo et al., 1996). In proto-*neu*-transgenic mice, Boggio et al (Boggio et al., 1998) found that prolonged administration of IL-12 delayed tumour onset and reduced tumour multiplicity in association with deficient peri- and intra-tumoural angiogenesis and infiltration of reactive cells. IL-12 showed anti-tumour activity when systemically administered after tumour challenge in mice vaccinated with IL-2 gene-transduced cells or in mice vaccinated with B7 costimulatory molecule-expressing tumour cells (Vagliani et al., 1996; Coughlin et al., 1995; Zitvogel et al., 1996). Recently, an adjuvant effect of IL-12 on vaccination with irradiated SCK tumour cells engineered to secrete granulocyte/macrophage colony-stimulating factor, has also been reported (Kurzawa et al., 1998).

I observed that immunisation with non-transduced tumour cells plus rIL-12 induced just a slight delay in tumour onset without the recruitment of anti-tumour T cells, suggesting that the anti-tumour response involves more than an enhancement of immunity signaled by the CpG motif (Fig. 7.3). Consistent with this observation data, obtained by Roman et al (Roman et al., 1997) using bacterial immunomodulatory DNA sequences, showed that these sequences activate the precise cytokine network required to induce an initial burst of IFN-γ in an antigen-independent fashion. In the presence of a protein antigen, the differentiation of naive $CD4^+$ T cells toward Th1 phenotype can be promoted, leading to a second burst of IFN-γ production, this time in an antigen dependent fashion. However, when I administered the recombinant murine IL-12 together with transduced tumour cells, I observed an enhanced effect. Indeed, a clear and significant delay in

tumour onset with 2 out of 12 immunised remaining tumour-free was observed at the end of the observation period (Fig. 7.3).

In agreement with the preferential Th1 response induced by *Ag38*-transduced cell vaccine, a population of $CD3^+CD8^+$ lymphocytes producing IFN-γ have been derived from spleen cells of vaccinated mice, which were able to proliferate *in vitro* in the presence of tumour cells (Fig. 7.6). These $CD3^+CD8^+$ lymphocytes produced IFN-γ in response to the recognition of N202.1A tumour cells. However, this production was not restricted to the recognition of HER2/neu oncoprotein, expressed on N202.1A cells, since IFN-γ production was detectable also in the presence of the non-HER2/neu expressing N202.1E cell line (Fig. 7.7).

The production of IFN-γ by the $CD3^+CD8^+$ lymphocytes and the strong inhibitory effect of this cytokine on the three different transgenic tumour cell lines suggest that induction of this cytostatic cytokine at the tumour site might represent at least one of the mechanism of action of the vaccine (Fig. 7.8).

The administration of an anti-IL-12 antibody did not significantly change IFN-γ production induced by vaccination with *Ag38*-transduced cells, indicating that additional factors related to Ag38 expression are responsible for the greater level of IFN-γ observed (Fig. 7.5).

Vaccination with transduced N202.1A tumour cells did not induce an humoral response against the vaccine. Moreover, unlike the situation observed in the melanoma model, in which a strong humoral response was detectable after challenge, no difference in the antibody profile was found between mice that developed spontaneous tumours and mice which were protected (Fig. 8.1).

A cell population able to respond and to proliferate in the presence of the tumour was derived from the spleens of the two mice completely protected from tumour

development. These cells passively transferred *in vivo* in the presence of tumour cells did not shown any inhibitory activity on tumour growth (Fig. 8.6). The ability to actively proliferate *in vitro* was a peculiarity only of spleen cells derived from these mice. Indeed, spleen cells derived from vaccinated mice which developed tumours were able to survive in *in vitro* culture for some weeks, but did not show an active proliferation, while spleen cells derived from unvaccinated mice did not completely respond to the tumour and survived *in vitro* at the most 2 weeks.

FACScan analysis indicated that these cells were $CD4^+$ T cells and, surprisingly, they produced IL-4 (Fig. 8.2B). The presence of cells producing a cytokine representative of a Th2 type subset in protected animals is apparently in contrast with the opposed Th1 phenotype observed in these mice immediately after vaccination. However, recent evidence has been derived which is opposed to the idea of an extreme separation between a Th1 status, as identified by IFN-γ production, and a Th2 status, as identified by IL-4 production. It is likely that regulatory systems exist to maintain a more appropriately balanced cytokine response which involves the simultaneous activation of both-types of regulatory cytokines. For example, a recent study by Hochrein et al (Hochrein et al., 2000) describes a feedback system mediated by IL-4. As well as the well-established positive feedback by which IL-4, as a Th2 cytokine, directly promotes Th2 differentiation from Th0 cells, these authors have found that IL-4 also has the potential to promote Th1 differentiation. This is an indirect effect via the action of IL-4 on dendritic cells to enhance the production and bioactivity of IL-12, the major Th1-inducing cytokine. Moreover, Klugewitz et al (Klugewitz et al., 2000) recently decribed a model of vaccination using the tumour-specific large T antigen against a murine kidney sarcoma, which induces a protective immunity dependent both on $CD8^+$ cytotoxic and on $CD4^+$ T-helper cells. These authors reported that the phenotype analysis of the $CD4^+$ T cells showed a general increase, as response to vaccination, both

in Th1 and Th2 cells and an increase in percentage of IL-4 producing cells only in vaccinated mice which rejected the tumour. It is not unlikely that the presence of increased $CD4^+$ T cells producing IL-4 is important for the maintenance of a tumour-specific CTL response (Santra et al., 1997) and of antigen-inducible IFN-γ expression among $CD8^+$ T cells in animals protected from tumour growth.

Consistent with these data, it has recently been reported on the identification of a melanoma-specific $CD4^+$ T cell clonal subset with a Th1 cytokine profile but releasing high amounts of IL-10 (Rivoltini et al. 2000). Since cells with such immunological features have been found to be present with high frequency in long-term surviving melanoma patients, it has been proposed that they are likely to play a role in mediating an effective long-lasting immunological control of tumour growth *in vivo*.

I observed that inhibitory factors released by the $CD4^+$ T cell population specifically inhibited the *in vitro* growth of different transgenic tumour cell lines (Fig. 8.3). This inhibition was not due to the release of IL-4, since this cytokine did not reveal any inhibitory effect on the growth of a transgenic cell line (Fig. 8.4). This inhibition apparently did not seem to be due to the eventual production of toxic factors in the conditioning of the *in vitro* culture of lymphocytes, since supernatant obtained by the culture of control spleen cells from untreated mice, maintained under the same conditions, did not reveal any toxicity. Therefore, it could be postulated that this effect might be mediated by other cytokines released by $CD4^+$ T cells. Unfortunately I was not able to investigate this issue during this thesis, because of the difficulty of obtaining large amounts of supernatant from the lymphocyte cultures, which were maintained in wells of small size and at high concentration. Moreover, at the end of my work I observed some alterations in the characteristics of these lymphocytes, probably due to the prolonged *in vitro* culture, such as the loss of dependence for their growth on the continued presence of tumour cells.

9.5 CONCLUSION: ADVANTAGES AND DISADVANTAGES OF THE APPROACH

Most investigations in the field of cancer gene therapy have been related to the antitumour effect of cancer vaccines with transfected cytokine genes, major histocompatibility antigens and co-stimulatory molecules (Dranoff et al., 1995; Porgador et al., 1995; Townsend et al., 1993). Some of these engineered tumour cells provide cytokines that bypass the need for helper cells, while the other modified tumour cells provide major histocompatibility molecules or co-stimulatory signals for direct stimulation of CD8 T cells or CD4 T cells.

Another approach to stimulating an immune response against tumours, based on transduction of a bacterial gene which represents a danger signal, has been evaluated in this thesis. Indeed, the immune response normally involves the integrated production of a variety of cytokines and the activation of different cell subsets that work in concert both locally and systemically. I reasoned that an enhancement of tumour immunity may be most effective if I used agents that can orchestrate the immune response, including cytokine production and cellular activation simultaneously, in a manner that reflects physiological responses. Furthermore, the activation of innate immune mechanisms, mediated by bacterial molecules, might induce a more prompt and rapid anti-tumour response. Compared to other strategies, such as transduction of cytokine genes or costimulatory genes, the use of bacterial genes might induce a wider and more physiological immune response.

In the light of the pivotal role of *M. tuberculosis* genes and their encoded proteins in linking innate and cell mediated adaptive immunity, these bacterial substances are promising candidates to be used as adjuvants for the development of effective

therapeutic or prophylactic tumour vaccines. Indeed, as above described, neonatal bacillus Calmette-Guèrin (BCG) vaccination has been demonstrated to confer protection against leukaemia and other childhood cancers (Grange et al., 1995), and recently a significant reduction in melanoma risk in people vaccinated with vaccinia virus or BCG was reported (Koelmel et al, 2000). These findings suggest that oncologic disease can be prevented by immunologic means but without a specific immunisation. The proposed bacterial transduced immunisation procedure could potentially be used for prevention in high-risk patients and in an adjuvant setting after surgery.

An important peculiarity of the vaccination protocol analysed in this thesis is that the protection is induced in mice by injections of irradiated tumour cells. This characteristic allows the translation of this vaccination protocol to human patients, in which the use of non-replicating tumour cells is mandatory. On the contrary, other approaches often require, to induce an efficient response, the administration of live engineered cells and a major number of injections. For example, it has been reported that B7-expressing tumour cells lose their ability to elicit systemic immunity on irradiation (Townsend et al., 1994; Cayeux et al., 1996; Katsanis et al., 1996). Other studies have shown that live cytokine-secreting tumour cells were superior to irradiated tumour cells in generating protective immunity. Most likely, irradiated non-replicating cells secrete a too limited amount of cytokine before dying. For example, it has been demonstrated by Dranoff et al (Dranoff et al., 1993) that irradiated B16 cells transduced to express either IL2, IL4, IL5, IL6, TNF or IFNγ failed to induce immunity to subsequent challenge with live parental B16 cells. A specific anti-tumour immunity was observed only using irradiated B16 cells transduced to express GM-CSF (Dranoff et al., 1993).

As with other transduced vaccines, when translated into the human application, some disadvantages limited the applicability of the strategy as designed in this thesis. The

creation of a cell vaccine based on transduction of the mycobacterial gene in tumour cells requires patient's cultured cells, transduction with problematic vectors, labor-intense development for scale-up to treat large number of patients. All these requirements are expensive, time-consuming and restricted to only a few oncologic centres.

9.6 SUMMARY

In an effort to increase immunogenicity of the recipient tumour, the *Mycobacterium tuberculosis Ag38* gene has been transduced in tumour cells. Transduced cells have been used as a cellular vaccine and their ability to elicit an anti-tumour response has been evaluated against both a transplanted tumour model and against a spontaneous tumour model. Protection against both tumours has been observed after only two vaccinations with irradiated transduced cells.

In both models, vaccination with transduced cells induced a preferential Th1 response.

In mice protected from transplanted melanoma tumour growth, a strong humoral response against tumour antigens with a prevalent Th1-dependent isotype was observed. This response was directed predominantly against endogenous retroviral antigens.

No humoral response was induced in mice protected from spontaneous mammary carcinoma development, while an activated $CD4^+$ T cell population producing IL-4 was derived *in vitro* from these mice.

In the light of the pivotal role of *M. tuberculosis* genes and their encoded proteins in linking innate and cell mediated adaptive immunity, these bacterial substances are promising candidates to be used as adjuvants for the development of effective therapeutic or prophylactic tumour vaccines. Furthermore, an enhancement of tumour

immunity may be most effective if agents are used that can orchestrate the immune response, including simultaneously cytokine production and cellular activation, in a manner that reflects physiological responses.

The elucidation of the clear mechanism induced by these bacterial substances in increasing the immune recognition of poorly immunogenic tumours and the evaluation of a strategy which employs these substances in a manner more easily translatable into human applications will be the future issues resulting from the work of this thesis.

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Sfondrini L., Morelli D., Bodini A., Colnaghi, M.I, Ménard, S., and Balsari, A.: "High level antibody response to retrovirus-associated but not to melanocyte lineage-specific antigens in mice protected from B16 melanoma". International Journal of Cancer, **83: 107-112** (1999).

Sfondrini L., Rodolfo M., Singh M., Colombo M.P., Colnaghi M.I., Ménard S. and Balsari A.: "Cooperative effects of *Mycobacterium tuberculosis* Ag38 gene transduction and IL-12 in vaccination against spontaneous tumor development in proto-neu-transgenic mice". Cancer Research, **60: 3777-3781** (2000).

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